A STUDY OF THE RELATION BETWEEN THE LIVER PROTEIN REGENERATION CAPACITY AND THE HEPATIC NECROGENIC ACTIVITY OF YEAST PROTEINS

J. A. GOYCO

Department of Biochemistry and Nutrition, School of Medicine, School of Tropical Medicine, University of Puerto Rico, San Juan

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The production of massive dietary hepatic necrosis in the rat by the use of yeast as the sole source of protein in rations deficient also in alpha-tocopherol has been reported by several investigators (Hock and Fink, '43; Glynn and Himsworth, '44; Goyco and Asenjo, '46; Gyorgy and Goldblatt, '49; Schwarz, '51a). However, irregularities have been observed in the frequency with which this nutritionally induced liver lesion is obtained. These discrepancies have been attributed to differences in experimental conditions, and, in particular, to the type of yeast fed to the animals.

Schwarz ('51b) has postulated the existence of a so-far unidentified liver-protective factor in some yeasts. The concentration of this factor seems to be very variable in different types of yeast. Lindan and Work ('51) found that bakers' and brewers' yeasts differ in respect to both growth-promoting and necrogenic properties. They indicated that the amino acid content of these two yeasts could explain the difference in growth-promoting properties; however, it could not account entirely for their different necrogenic activity. Gyorgy et al. ('50) found that by feeding a British brand of bakers' yeast, as the sole source of protein, hepatic necrosis occurred with great regularity. while when an American brand of brewers'

yeast was fed, no liver necrosis could be obtained. These latter investigators proposed the hypothesis that the so-called dietary hepatic necrosis is due to an unidentified toxic factor rather than to a deficiency state and indicated that the possible roles of α-tocopherol or cystine or both could be that of detoxifying agents.

Goyco and Asenjo ('47, '54) observed, while carrying out studies on the nutritive value of different types of yeasts, that the incidence of liver necrosis was more frequent in the animals receiving the yeast proteins possessing the lowest biological and growth-promoting values.

It is the purpose of the present investigation to correlate the liver protein regeneration capacity of two different types of yeasts with their respective necrogenic activities and to study also the effect of different added supplements.

EXPERIMENTAL

Experiment on liver protein regeneration by yeast. The procedure employed was that described by Harrison and Long ('45), with the following modifications: in the experimental rations the 8% yeast was replaced by a mixture of pure vitamins, offered daily (0.5 ml) to the animals. The composition of this mixture in milligrams per milliliter was: biotin, 1; folic acid, 1; thiamine, 6; pyridoxine, 6; riboflavin, 10; Ca-pantothenate, 20; nicotinic acid, 20; p-aminobenzoic acid, 20; inositol, 200; choline hydrochloride, 3460. One drop of a solution of α-tocopherol acetate in corn oil (1 mg/drop) was given every other day. The stock synthetic ration consisted of: casein (vitamin-free), 20%; cornstarch, 50%; hydrogenated vegetable oil,[1] 24%; cod liver oil, 2%; salt mixture no. 2 U.S.P., 4%. The animals were fed 15 to 16 gm of ration daily, equivalent to a caloric intake of about 25 Cal. per 100 gm of body weight.

After a preliminary period of 7 days on the stock synthetic ration the rats were fasted for 48 hours and then fed for 4

[1] Crisco.

days the synthetic rations containing the yeasts or the casein. The rations were made to contain 9 or 18% protein and all were isocaloric with the stock ration. The composition of the rations was the same, the only variable was the type of protein used. Two yeasts were studied: a dry brewers' yeast [2] and a *Torula utilis* dry yeast locally produced. Supplementation of both yeasts with DL-methionine and *Torula* yeast with L-cystine was also investigated. In addition, the supplementary action of brewers' yeast on *Torula* yeast was investigated. In all trials, groups of at least 5 male albino rats (Wistar — S.T.M.[3] strain) weighing approximately 300 gm were used.

After the 4 days refeeding period, the animals were anesthetized [4] and exsanguinated through the abdominal aorta in order to ensure minimum and approximately uniform quantities of blood in the livers. The whole liver was removed, washed in running water and blotted dry with filter paper. It was then weighed in a small porcelain casserole and dried to constant weight at 95°C. The dried liver was then ground in an Osterizer to a fine powder and duplicate samples taken for total nitrogen determination. Nitrogen was determined by the micro-Kjeldahl method.

Both control and fasted animals were examined. The control group consisted of rats which had been on the stock synthetic ration for 7 days and sacrificed without submitting them to a fasting period. The fasted group consisted of rats which after having been on the stock synthetic ration for 7 days were fasted for 48 hours and then sacrificed. The results of these experiments are summarized in table 1.

Experiment on necrogenic properties of yeast. For this series of experiments weanling (21 days) rats from our stock colony were used. Litters were evenly distributed among the experimental rations. The yeast samples used were the same ones utilized for the experiment on liver protein regeneration. The composition of the rations was: yeast protein, 9 or 18%;

[2] Fleischmann, type 2019.
[3] School of Tropical Medicine.
[4] Pentothal sodium (Abbott).

lard, 5%; salt mixture (U.S.P. no. 2), 4%; cod liver oil, 1%; and cornstarch to make up 100%. A supplement of a mixture of pure vitamins (already described) was given to each animal every other day. The rations were fed ad libitum. Post mortem examinations were made of all of the animals when they died or at the end of experimental period (10 weeks). The livers were examined, removed, weighed, dried and kept for further chemical analyses. The results of this experiment are summarized in table 2.

TABLE 1

Regeneration of liver protein following a 48-hour fast using rations containing two types of yeast

RATION	PROTEIN IN RATION (N × 6.25)	NO. OF RATS	BODY WEIGHT CHANGE DURING REFEEDING	LIVER PROTEIN (N × 6.25)	LIVER PROTEIN INCREMENT AS COMPARED TO CONTROLS
	%		*gm*	*mg per 100 gm body weight*	%
Non-fasted controls	18	12	..	737 ± 1.7 [1]	..
Fasted controls	..	12	..	581 ± 1.4	..
Casein	18	10	23	725 ± 2.0	92
Torula yeast	18	10	17	612 ± 2.9	20
Torula yeast	9	5	15	593 ± 2.0	8
Torula yeast + 0.5% DL-methionine	18	10	35	725 ± 1.9	92
Torula yeast + 0.5% L-cystine	18	5	35	731 ± 4.9	96
Brewers' yeast	18	10	29	737 ± 2.6	100
Brewers' yeast	9	5	21	625 ± 3.1	28
Brewers' yeast + 0.5% DL-methionine	18	10	32	756 ± 2.6	112
⅔ Torula yeast + ⅓ brewers' yeast	18	5	32	731 ± 3.6	96

[1] Mean ± standard error of the mean.

RESULTS AND DISCUSSION

The restoration of the liver protein lost during fasting by feeding a protein-containing food has been suggested as a basis for the assay of the nutritive value of proteins. Campbell and Kosterlitz ('48) found that the liver nitrogen in the rat expressed in terms of body weight, was a function of the amount, as well as of the quality of protein fed. They offered

TABLE 2

Incidence of liver necrosis on rations containing two types of yeast

RATION	PROTEIN IN RATION (N × 6.25)	NO. OF RATS	AVERAGE GAIN IN WEIGHT FOR FIRST 3 WEEKS [1]	NO. OF RATS WITH MASSIVE LIVER NECROSIS	INCIDENCE OF LIVER NECROSIS
	%		gm		%
Casein	18	6	43	0	0
Torula yeast	9	16	0	16	100
Torula yeast	18	16	28	10	62
Torula yeast + 0.5% DL-methionine	18	9	64	0	0
Torula yeast + 0.5% L-cystine	18	5	63	0	0
Brewers' yeast	9	16	17	16	100
Brewers' yeast	18	15	57	0	0
⅔ Torula yeast + ⅓ brewers' yeast	18	5	51	0	0

[1] The average initial weight for the animals used in this experiment was 36 ± 1.25 gm.

the suggestion that the method may lend itself to a rapid assessment of the biological values of proteins.

The results (table 1) show that under our experimental conditions, the loss in liver protein after a 48-hour fast was approximately 21% of the original protein content. This value is lower than the one found by Harrison and Long ('45), 32%; however, our value (737 mg per 100 gm body weight) for the non-fasted control group is in good agreement with the one

reported by them (120 mg N $\times$ 6.25 = 750 mg protein per 100 gm body weight). The loss of body weight during the 48-hour fast was very uniform in all groups of rats, the average value being 23 gm. This value is very similar to that reported by the above authors, namely 24 gm. As can be seen in table 1, in all the rations, except the 9 and 18% *Torula* and 9% brewers' yeast, the animals regained the body weight lost during the fasting period and in most cases this value was surpassed.

The animals that were realimented with a ration containing casein at an 18% protein level showed an excellent (92%) regeneration of the liver protein. This group was included to serve as a basis for comparison of the results obtained with the yeast-containing rations.

The results obtained when the two yeasts were supplied at 9 and 18% protein levels indicate that there was a very small regeneration of the liver protein in the animals receiving the *Torula* yeast at both levels and also with the brewers' yeast at 9% level. On the other hand, with the brewers' yeast protein at 18%, the liver protein increased to the prefasting level. When both yeast rations were supplemented with 0.5% of DL-methionine and the *Torula* ration with 0.5% L-cystine, the liver protein increased to values equal to or higher than those observed in the prefasting levels. Both results agree with those found by Goyco and Asenjo ('49) when the biological value of these same yeasts was determined using the nitrogen balance method. With the ration containing a mixture of both yeasts ($\frac{2}{3}$ *Torula* and $\frac{1}{3}$ *brewers'*) an almost complete regeneration of liver protein was observed. This indicates an excellent supplementation of these two yeast proteins.

It will be seen from the results (table 2) of the experiment on the necrogenic properties of these yeast rations that the *Torula* yeast is necrogenic at both levels. In contrast, the brewers' yeast proved to be necrogenic only at the 9% level and non-necrogenic at the higher level. The rate of growth of the animals during the first three weeks of the experimental period was, in the case of the 9% brewers' yeast ration, considerably higher than in the case of the 9% *Torula* ration.

While no gain in body weight was shown during this initial period by the animals fed *Torula* yeast, those fed brewers' yeast made an average gain of 17 gm. However, the average survival time of the rats of the *Torula* yeast group was 42 days while that for those fed brewers' yeast was only 32 days.

The necrogenic activity of the *Torula* yeasts when fed at 18% level was somewhat lower (62% incidence) during the experimental period of 10 weeks. This percentage might have been higher if the experimental period had been longer but for the purpose of this study this was not considered necessary. No necrosis was observed in the animals receiving the casein ration.

Both DL-methionine and L-cystine when fed at a 0.5% level together with the *Torula* yeast protected all the animals from developing liver necrosis. Both of these amino acids, as can be seen in table 2, improved significantly the rate of growth of the animals on the *Torula* yeast rations.

Full protection against liver necrosis was also obtained when both yeasts were fed together. In this case, also, the improvement in the growth rate of these animals was remarkable when compared with that of those fed *Torula* alone.

These results seem to point to the fact that brewers' yeast protein at an 18% level is capable of restoring maximum liver protein stores in the depleted animals and also protects against liver necrosis. On the other hand, *Torula* yeast at this same level is incapable of accomplishing either one of these two goals. However, when brewers' yeast was fed at the 9% level, only a small fraction of the protein lost during the fasting period was regenerated and the animals developed liver necrosis. It is apparent that a close parallelism exists between liver protein regeneration capacity and the necrogenic activity of a yeast ration.

When the necrogenic 18% protein *Torula* yeast ration was supplemented with DL-methionine and L-cystine, liver necrosis was prevented in the experimental animals. Also the liver protein regeneration capacity of these rations was so much

improved that all depelted animals regained their normal protein stores in the liver.

This confirms the observations previously made that these two amino acids also improved the biological and growth-promoting values of the *Torula* yeast protein (Goyco and Asenjo, '49, '54).

The results obtained when a mixture of *Torula* and brewers' yeasts is fed are difficult to explain on the basis of amino acid supplementation, considering that both yeasts have a similar amino acid make-up, in particular in regard to their content of sulphur-containing amino acids.

Factor 3 of Schwarz could be responsible for the non-necrogenicity of the ration containing the 18% mixed yeast proteins. Whether Factor 3 is also responsible for the excellent liver protein regeneration capacity of this mixture containing 6% brewers' and 12% *Torula* yeast proteins, is something that should be determined in the future.

At the present time we are studying the effect that a supplement of vitamin E might have on the liver regeneration capacity of *Torula* yeast protein. Adult rats depleted of vitamin E are being used, as we believe that the presence of even small amounts of this vitamin in the animal tissue will tend to mask the effect which could be produced by the addition of a vitamin E supplement to the rations.

SUMMARY

Liver protein regeneration and hepatic necrogenic activity of two different types of yeasts (a brewers' and a *Torula* yeast) have been studied. The results show a close parallelism between these two activities.

The *Torula* yeast was found to be necrogenic at both levels used (9 and 18% protein) and also was incapable of restoring liver protein in the depleted animals.

The brewers' yeast behaved similarly at the lower level but at the 18% protein level was found to be non-necrogenic and

also capable of restoring maximum liver stores of protein in the depleted animals.

Both DL-methionine and L-cystine when fed at a 0.5% level together with the *Torula* yeast protected all of the animals from developing liver necrosis and also induced a complete regeneration of the liver protein in the depleted animals.

When both yeasts were fed together ($\frac{1}{3}$ brewers' and $\frac{2}{3}$ *Torula* yeasts) full protection against liver necrosis as well as optimum liver protein regeneration was obtained.

ACKNOWLEDGMENT

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NUTRITIONAL STUDIES ON THE BLOWFLY, PHORMIA REGINA (MEIG.)[1]

A. J. McGINNIS,[2] R. W. NEWBURGH AND VERNON H. CHELDELIN
Department of Chemistry and the Science Research Institute
Oregon State College, Corvallis

ONE FIGURE

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Studies on insect nutrition have been many and varied as evidenced by reviews such as those of Uvarov ('28), Craig and Hoskins ('40), and Trager ('47). However, it was not until recent years that detailed studies of specific nutritional requirements for any one species were accomplished.

The first insect species reared on a chemically defined diet was *Drosophila melanogaster* (Schultz et al., '46). Since then several other species also have been reared in this manner. Among these are the mosquito *Aedes aegypti* (Trager, '48), the cockroach *Blattella germanica* (House, '49), the spruce budworm parasite *Pseudosarcophaga affinis* (House, '54), and the fly *Calliphera erythrocephala* (Meigen) (Sedee, '54).

Hill et al. ('47) reported a method of rearing the blowfly, *Phormia regina,* under aseptic conditions on a simple artificial

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A preliminary report was presented before the 19th meeing of the American Institute of Nutrition, San Francisco, California, April, 1955. While this work was in progress Brust and Fraenkel ('55) also reported on the nutritional requirements for the larvae of the blowfly.

[2] On Educational Leave, Division of Entomology, Science Service, Canada Department of Agriculture. Present address, Science Service Laboratory, Lethbridge, Alberta, Canada.

TABLE 1

Composition of diets used in nutritional studies with P. regina

CONSTITUENTS	DIET									
	A	B	C	D	E	F	G	H	I	J
	%	%	%	%	%	%	%	%	%	%
Vitamin-free casein	26	29	23	19	23	19	23	19		
Yeast extract	3	3	3	3	3	3	3	3	3	3
Glucose			6	10						
Sucrose					6	10				
Soluble starch							6	10		
Enzyme hydrolyzed casein									29	
Acid hydrolyzed casein										29
Agar	1	1	1	1	1	1	1	1	1	1
Water (containing supplements)	70	67	67	67	67	67	67	67	67	67
	Supplements (milligrams per gram of medium)									
DL-tryptophan									5.8	5.8
Cholesterol	0.78	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
NaCl	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
KCl	0.015	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
$CaCl_2$	0.015	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
$NaHCO_3$	0.015	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
$NaH_2PO_4 \cdot H_2O$	0.41	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
K_2HPO_4	0.35	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30

Final volume per tube = 6 ml.

medium, although details concerning the essential nutrients are lacking. The present paper reports on a chemically-defined diet adequate to support development of *P. regina* from egg to adult under aseptic conditions. Certain of the essential metabolites for this organism have been established.

MATERIAL AND METHODS

The insects used throughout this study were the progeny of a wild population trapped in the vicinity of Corvallis, Oregon. Succeeding generations of flies were reared on a purified medium (diet A) modeled after that of Hill et al. ('47). The adults were maintained on sucrose cubes and water. When oviposition was desired, either fresh liver or kidney was placed in the cage. Oviposition usually followed within 24 to 48 hours. The colony was maintained in a rearing room at 80 ± 2°F. The nutritional studies encompassed the complete life cycle of the blowfly. This comprised, in order of development, the egg, larva (maggot), pupa and adult.

The final composition of a number of diets used in the nutritional studies is given in table 1.

The complete medium was compounded by combining three separate fractions. The first fraction consisted of a solution of the different salts in distilled water. A cholesterol suspension containing 50 mg/ml as described by House ('49) constituted the second fraction. The third, a mixture of the appropriate dry ingredients — casein, yeast extract, agar, carbohydrate and tryptophan — formed the dietary variable.

In preparing the diets, the liquid components, formed by combining the salts solution and cholesterol suspension were first introduced into the container. The dry components were then added and thoroughly mixed. The medium described by Hill et al. ('47) contained lanolin. Cholesterol at a level of 0.67 mg/gm of medium was used in place of lanolin.

Diet A was used for bulk rearing. One hundred grams of this diet were mixed in a 500 ml Erlenmeyer flask and sterilized at 16 pounds/sq. in. of steam pressure for 15 minutes.

This quantity of medium permitted development of approximately 400 blowflies.

In nutritional studies 6-inch culture tubes were used as growth chambers. The appropriate components were placed in the tubes which were then covered with aluminum caps and sterilized by autoclaving as above. After removal from the autoclave, the tubes were set at a 30-degree angle and allowed to cool. In all experiments using diets A through J, 6 gm of medium were placed in each tube, and at least three tubes used for each component or other variable in the diet.

When a component was tested at a series of different concentrations it was added as an aqueous solution. To maintain a constant composition, half quantities of a double strength solution of salts and cholesterol were used. Water was added as necessary to give the correct final concentration.

In later experiments, a purified amino acid mixture was substituted for the casein of the diets listed in table 1. The composition of the amino acid diet is given in table 2.

In the preparation of the amino acid mixture, appropriate quantities of the 18 amino acids were weighed out on an analytical balance, placed in a mortar and ground together thoroughly. Seventy-five milligrams of this mixture and 50 mg of agar were added to each culture tube. Individual vitamin solutions were freshly prepared for each experiment at such concentrations that the addition of 0.1 ml of the solution sufficed for the final volume of medium (2 ml) in each tube. The three remaining components were combined in solution form immediately prior to use. The double strength salts solution used in previous experiments was the mineral source. The stock cholesterol suspension used was that described previously. The ribonucleic acid solution was freshly prepared for each experiment by dissolving 200 mg of yeast ribonucleic acid in 4 ml of 2N NaOH. One milliliter of the solution, consisting of 44 parts of salts solution, two parts of cholesterol suspension, and 4 parts of ribonucleic acid solution, was added to each culture tube.

The contents of the tubes were thoroughly mixed by shaking prior to sterilization at 16 pounds pressure for 15 minutes. After removal from the autoclave, slants were prepared as described previously. The final pH was approximately 5.6. If the pH deviated greatly from this value the agar hydrolyzed and the medium failed to solidify.

TABLE 2

Composition of chemically-defined diet for P. regina

AMINO ACIDS	CONCENTRATION	VITAMINS	CONCENTRATION
	mg/ml		*μg/ml*
DL-Alanine	2.58	Thiamine	5
L-Arginine	1.06	Riboflavin	15
DL-Aspartic acid	3.06	Pyridoxine	15
L-Cystine	0.36	Inositol	100
L-Glutamic acid	5.48	Nicotinic acid	15
Glycine	0.70	Choline chloride	100
L-Histidine	0.70	Ca pantothenate	15
Hydroxy-L-proline	0.48	*p*-Aminobenzoic acid	15
DL-Isoleucine	3.06	Folic acid	5
L-Leucine	2.80	Biotin	0.025
L-Lysine	1.76		
DL-Methionine	1.88		
DL-Phenylalanine	2.80		
L-Proline	2.00		
DL-Threonine	1.88		
DL-Tryptophan	0.94		
L-Tyrosine	1.64		
DL-Valine	3.30		

	CONCENTRATION *mg/ml*
Ribonucleic acid	1.0
Cholesterol	1.0
Inorganic salts	2.4
Agar	25.0

Final volume per tube = 2 ml.

Sterilization of the eggs was accomplished by first placing the fresh egg masses in a 2% NaOH solution until the egg separated (about 15 minutes). The NaOH was decanted off, and the eggs were rinsed twice with 70% ethanol to remove the remaining alkali. They were then immersed in 70% ethanol for 15 minutes, and transferred aseptically to the culture tubes on the tip of a small alcohol-sterilized camel's hair brush. Approximately 5 eggs were placed in each tube. The tubes were incubated in a 30°C. water bath and the development was recorded by measuring the larval length.

RESULTS

Effect of adding carbohydrate to the diet. The standard diet, B, to which all others were compared, is shown in table 1. Blowflies reared on this medium reached larval maturity in 8 days and emerged as adults in 16 days.

Since the diet contained no carbohydrate, it was of interest to determine the effect of its inclusion. The substitution of glucose for casein in the diet resulted in a retardation of development. On diet C (6% glucose) growth was reduced approximately 15%. With diet D (10% glucose), mortality of young underdeveloped larvae was very high. When glucose was present, the normally light colored medium turned brown during sterilization. This type of reaction has been reported by Lemonde and Bernard ('51) to produce a diet toxic for *Tribolium confusum.*

In diets E, F, G, and H, which included sucrose and soluble starch, no browning occurred. This was presumably due to the absence of reducing sugar. At the 6% level, sucrose permitted more rapid growth and less mortality than did glucose. Six per cent of starch in the diet produced a response similar to that of sucrose. When diets containing 10% of carbohydrate (glucose, sucrose or starch) were tested it was found that only the starch diet permitted growth. These findings, which are similar to those of Brust and Fraenkel ('55), suggest that some other factor besides browning is responsible for the slower growth. Curves showing the larval response on

different carbohydrate-containing diets are presented in figure 1. This figure further illustrates the method by which growth was followed and the growth curves obtained.

Effect of hydrolyzed casein in the diet. In an effort to approximate more closely a chemically defined medium for this organism, hydrolyzed casein was tested, both as acid and enzyme digests (diets I and J). Development on these media

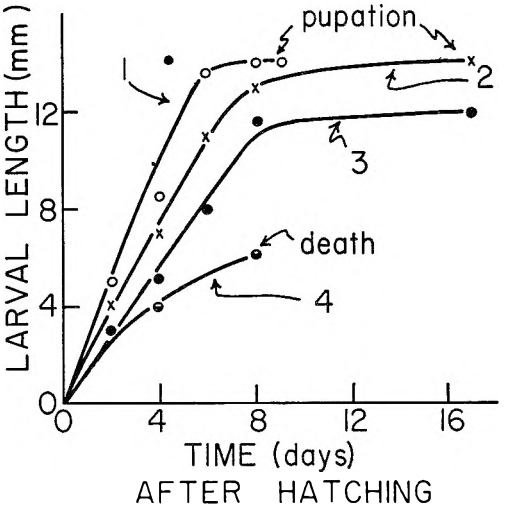

Fig. 1 Effect on *P. regina* development of replacing casein by different carbohydrates in the diet.

See table 1 for composition of diets.
Curve 1 — Diet B (Basal).
Curve 2 — Diets E, G, and H (Basal + 6% sucrose, 6% starch or 10% starch).
Curve 3 — Diet C (Basal + 6% glucose).
Curve 4 — Diets D and F (Basal + 10% glucose or 10% sucrose).

was very poor. This suggested either the release of some toxic component or the absence of essential peptides in the hydrolyzed casein. Diets B and I were combined in different proportions in an effort to overcome this inhibition. Growth equivalent to that on the standard ration, B, was obtained only when 70% or more of diet B was present. Further, the ingredients of diet I in the mixed medium were actually inhibitory; better growth was obtained when the casein content of

diet B was reduced by half than when diet B was mixed with an equal quantity of diet I. Similar results have been reported by Stokstad ('40) with growing chicks.

This pointed further to the release of an inhibitory factor into the medium. The most likely compounds involved would seem to be the amino acids; certain of them have been shown to inhibit growth of microorganisms (Snell and Guirard, '43), and D-serine is known to be toxic to Drosophila (Hinton et al., '51). In view of these observations, glycine, DL-serine, DL-threonine, L-tyrosine, and L-glutamic acid were tested in culture tubes containing 6 gm of diet B. L-Glutamic acid appeared to be slightly inhibitory at a concentration of 100 mg per tube. No inhibition was noted when glycine, DL-threonine or L-tyrosine were included at the rate of 50 mg per tube. In contrast, 10 mg of DL-serine completely inhibited larval growth and caused death shortly after hatching. When the serine isomers were tested individually, it was found that only the D-form was inhibitory; 5 mg was as effective as 10 mg of the racemic mixture.

In view of the known competition between serine and alanine, the latter was tested as a reversing agent. It was found that 30 mg of D-alanine could reverse the inhibition by 10 mg of DL-serine, whereas the L-isomer was ineffective.

The fact that toxicity in serine is conferred by the D-isomer eliminates it as the toxic component of enzyme-hydrolyzed-casein and probably also of acid-hydrolyzed casein since these digests should yield only L-amino acids. It seems safe to conclude that some other unknown factor or factors is causing the inhibition of growth on the enzyme-hydrolyzed-casein diet. That this same factor is responsible, at least in part, for the inhibition noted on the acid-hydrolyzed casein diet seems probable.

The inhibitory effect of the dipeptide, glycyl-DL-serine was tested by addition of 30 mg to the standard assay tube. This corresponds to a molar concentration twice that at which DL-serine is known to completely suppress development. However, no retardation in larval development was noted as shown

in table 3. In this same experiment the effect of N-acetyl-DL-alanine as a reversing agent was tested. To each tube containing 10 mg of DL-serine were added 88 mg of this N-substituted amide (corresponding to 60 mg of DL-alanine). However, only slight reversal was observed. In order to study further the specificity of D-serine toxicity and D-alanine reversal it was felt advantageous to test the antibiotic D-oxamycin,[3] a cyclic form of serine (Kuehl et al., '55), and β-aminoxy-DL-alanine ethyl ester.[3] The results are shown in table 4. Neither

TABLE 3

Effect of glycyl-DL-serine as a growth inhibitor, and of N-acetyl-DL-alanine in reversing D-serine inhibition

TREATMENT	LARVAL LENGTH (mm)				
	Days after inoculating				
	2	3	4	6	9
Diet B	4	7	10	14	14
Diet B + 10 mg DL-serine	3	dead	..	..	..
Diet B + 30.8 mg glycyl-DL-serine	4	7	10	14	14
Diet B + 10 mg DL-serine + 88 mg N-acetyl-DL-alanine	3	4	4	4	5
Diet B + 10 mg DL-serine + 60 mg DL-alanine	4	6	9	13	14

of these compounds exerted any influence on the growth of *P. regina.*

Development on chemically-defined medium. In the initial studies with a chemically-defined medium, the diet was modeled after that described by House ('54). Certain modifications were made and a diet was finally compounded (table 2) which permitted development from egg to adult.

The adequacy of the chemically-defined diet was demonstrated by the fact that larval growth compared favorably with that on the standard casein diet B. Pupation occurred 4 days later on the chemically-defined diet but the length of

[3] D-Oxamycin and β-aminoxy-DL-alanine ethyl ester were supplied by Merck and Co., Inc., by the courtesy of Dr. Karl Folkers.

pupation to emergence was similar. It is of interest that in the studies of Brust and Fraenkel ('55), nucleic acids, adenine, guanine or uracil apparently retarded emergence. The delay in metamorphosis on the present diet may thus have been due at least in part to the ribonucleic acid component.

Specific amino acid requirements for this insect were studied by the deletion of single amino acids from the mixture. No other amino acid was added to the medium as a nitrogen replacement, therefore slight variations existed in the total nitrogen contents of these diets.

TABLE 4

Effect of D-*oxamycin and β-aminoxy-*DL-*alanine ethyl ester on the growth of P. regina*

	ADDITIONS TO DIET B						
	None	D-serine	D-serine + β-aminoxy-DL-alanine ethyl ester		β aminoxy-DL-alanine ethyl ester	D-oxamycin	
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
		5	5	30	30	2	5	10
Days of seeding to								
Death	..	4	4		..	..	..	..
Pupation	7	..	..		9	9	7	8
Emergence	11	..	..					

The results from this study, shown in table 5, indicate that 10 amino acids are essential for growth: arginine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, threonine, tryptophan and valine. When any one of this group was omitted from the diet, the larvae died shortly after hatching without increasing appreciably in size.

The nitrogen content of this chemically-defined diet was only about 10% of that of the casein diet. It seemed likely that better growth could be obtained if the nitrogen content of this artificial medium were increased. However, when the amino acid mixture was used at a level of 870 mg per tube, which gave

TABLE 5

Development of P. regina on purified diets when single amino acids were omitted

DIET	DAYS FROM SEEDING TO			DIET	DAYS FROM SEEDING TO	
	Death	Pupation	Emergence		Pupation	Emergence
Complete [1]	..	12–14	19–20			
Complete + DL-serine	4	..	..			
A. Essential amino acids				*B. Effect of omission of other amino acids*		
Complete minus L-arginine	4	..	..	Complete minus DL-alanine	14	19
Complete minus L-histidine	4	..	..	Complete minus DL-aspartic acid	14	20
Complete minus DL-isoleucine	9	..	..	Complete minus L-cystine	14	20
Complete minus L-leucine	4	..	..	Complete minus hydroxy-L-proline	14	19
Complete minus L-lysine	4	..	..	Complete minus DL-methionine	14	19
Complete minus DL-phenylalanine	4	..	..	Complete minus L-glutamic acid	14–19	
Complete minus L-proline	6	..	..	Complete minus glycine	13	
Complete minus DL-threonine	4	..	..	Complete minus L-tyrosine	20	
Complete minus DL tryptophan	6	..	..			
Complete minus DL-valine	4	..	..			

[1] Complete diet is that of table 2.

a nitrogen content equal to that in diet B, complete inhibition of larval development resulted. Different levels of the amino acid mixture were tested and it was found that concentrations in excess of 60 mg per milliliter were inhibitory.

The first experiments to determine which vitamins were essential in the diet were conducted using a vitamin-free casein-ribonucleic acid-agar mixture as the basal diet. To the assay tubes were added solutions of all vitamins except the one under examination. Two milliliters of double strength inorganic salts solution containing 2 mg of cholesterol per milliliter were added to each tube. Two grams of the basal ration were then added with thorough mixing and sterilized as described previously. (Total volume $\doteq$ 4 ml.) Thiamine, nicotinic acid, pantothenic acid and choline were shown to be essential. However, owing to the possibility that the casein contained trace amounts of vitamins, the experiment was repeated using the amino acid mixture (table 2) as the nitrogen source. Results from this study confirmed the essentiality of the above 4 components and showed that riboflavin and pyridoxin were also necessary. In the absence of folic acid and biotin, pupation occurred but the flies did not emerge even after 26 days. *p*-Aminobenzoic acid and inositol were not required.

DISCUSSION

Inhibition of larval growth by serine has been observed by other workers (Schultz et al., '46; Hinton et al., '51). However, the finding that only the D-isomer is inhibitory and that D-alanine may restore normal growth, may suggest similar antagonisms in other species where DL-alanine has appeared to stimulate development.

Results obtained with the peptides, glycyl-DL-serine, D-oxamycin, N-acetyl-DL-alanine and β-aminoxy-DL-alanine ethyl ester suggest two points of significance. First, because these peptides or derivatives did not show the toxic effect of D-serine nor the reversing effect of D-alanine it appears that these compounds are not hydrolyzed in the insect body. Second, the free

amino group of each amino acid is essential, evidently, for the inhibition or its reversal. As yet, however, there is no information relative to the site of action of D-serine. Assuming that alanine racemase is present in this organism, it is possible that serine may inhibit the interconversion of alanine isomers.

Observations of larval development on several different media suggest that the growing larvae may be sensitive to the osmotic pressure of the medium. Larvae failed to grow on diets containing either 10% of glucose or sucrose, yet matured on a diet containing 10% of soluble starch. Similarly, diets containing either acid- or enzyme-hydrolyzed casein as the nitrogen source failed to support growth while the same diet containing whole casein served as the standard medium throughout the study. With the chemically-defined medium, growth was almost equivalent to that on the standard diet provided the amino acid concentration did not exceed 60 mg per milliliter. Thus, although the diets varied considerably in composition it was noticeable that the media which failed to support larval growth were those which had higher osmotic pressures.

In this study a diet which supported development from egg to adult was considered to be nutritionally adequate. However the reproductive phase of the adult was not studied. Rasso and Fraenkel ('54) have shown that a protein source is necessary in the diet of the adult *P. regina* female if ovarian maturation is to occur. It is possible, therefore, that although the chemically-defined diet may be adequate for larval development and metamorphosis it may be unable to meet the needs of the adult female for oviposition. Thus, whether this species can complete its life cycle on the chemically-defined diet described, is at present unknown.

SUMMARY

The diet proposed by Hill et al. ('47) for *P. regina* was modified by substituting crystalline cholesterol for lanolin.

The casein content of the standard diet was high (29%); however, replacement of a part of the casein by carbohydrate

resulted in slower growth and metamorphosis. This effect progressed in the direction starch < sucrose < glucose, with complete growth failure on a diet containing 10% of glucose.

Neither enzyme-hydrolyzed casein nor acid-hydrolyzed casein plus tryptophan was able to replace the whole casein in the diet.

It was demonstrated that 0.83 mg of D-serine per gram of medium caused complete inhibition and resulted in early death of the small immature larvae. This inhibition could be completely reversed by 5 mg of D-alanine. The free amino group appeared to be required in both amino acids to evoke this response.

A chemically-defined diet was prepared on which the blowfly developed from egg to adult. Ten amino acids proved to be essential: arginine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, threonine, tryptophan, and valine. Six vitamins were shown to be necessary in the diet: thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenic acid and choline.

The possibility was considered that the developing larvae are sensitive to high osmotic pressure in the medium.

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DENTAL CARIES IN THE ALBINO RAT IN RELATION TO THE CHEMICAL COMPOSITION OF THE TEETH AND OF THE DIET

II. VARIATIONS IN THE CA/P RATIO OF THE DIET INDUCED BY CHANGING THE PHOSPHORUS CONTENT

WINFREY WYNN, JOHN HALDI, KATHERINE DICKEY BENTLEY AND MARY LOUISE LAW

WITH THE TECHNICAL ASSISTANCE OF DORIS RAMSEY

Department of Physiology, Division of Basic Sciences in the Health Services, Emory University, Emory University, Georgia

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Recent studies appear to lend support to the ideas expressed some years ago by Mellanby ('18, '23) that resistance to dental caries is related to the chemical structure of the teeth, which in turn is determined by dietary influences at the time of tooth development. Sobel and Shaw (Sobel, '52) found that the molars of rats that had been fed a low calcium - high phosphorus diet in early life were considerably less susceptible to caries than the teeth of rats that had been fed a high calcium-low phosphorus diet. Earlier studies (Sobel, Rockenmacher and Kramer, '45a, b) had shown that the composition of the diet is reflected in the blood serum and that the composition of the bone is related to that of serum. Subsequent investigations (Sobel and Hanok, '48) on the growing incisor teeth of rats gave results similar to those obtained in the studies on bone.

On the basis of these observations Sobel has proposed the hypothesis that the composition of the diet is reflected in the blood; that there is a relationship between the inorganic composition of teeth and of the fluid from which the tooth salts

precipitate and that the composition of this fluid is in turn related to the blood serum. According to this hypothesis the mineral composition of the developing tooth can be influenced by the composition of the diet. Furthermore, Sobel ('55) believes that resistance to dental caries is related to the chemical composition of the tooth and in turn to the influence of diet during tooth development.

Various studies with radioactive isotopes during the past decade have shown that even after calcification of the tooth has been completed there are mechanisms for the transport of elements from the saliva to the dentine by way of the enamel and from the blood to the enamel by way of the dentine. The earlier evidence has been reviewed by Armstrong ('42). More recently Sognnaes, Shaw and Bogoroch ('55) have provided both confirmatory and additional evidence to show that radioactive phosphorus, iodine, potassium, sodium and water penetrate through the teeth not only from the external surface inward but also in the opposite direction outward.

These observations suggest the possibility that even after eruption the composition of the teeth may be affected by dietary influences and thereby be made more or less resistant to dental caries. The present study was undertaken to investigate this possibility. The Ca/P ratio of the diet was varied to determine the effects of such variation on the cariogenicity of the diet and on the chemical composition of the molar teeth after calcification had been completed.

EXPERIMENTAL PROCEDURE

There were two sets of experiments: one to determine the extent of caries that would develop on diets with various Ca/P ratios; the other to determine whether or not these same diets would have any effect on the chemical composition of the teeth.

Experiment I. Albino rats were selected in groups of four litter mates when 23 days old. These rats were desalivated in order to develop caries to an appreciable extent within a 70-day period. Each animal in a group was placed on the same basic cariogenic diet but with a different Ca/P ratio. The

diets consisted of 64% sucrose, 20% casein, 8% fat, 4% yeast and liver extract and 4% salt mixture with vitamin supplements (Haldi and Wynn, '52). The calcium content of all 4 diets was maintained at a constant level of 0.5% of the entire diet. This amount of calcium is generally considered to be adequate for the growth and well-being of the rat (McCoy, '49). The Ca/P ratios in the 4 diets were 1: 0.5, 1: 1, 1: 2 and 1: 3. No phosphate was added to diet no. 1; consequently the phosphorus in this diet was derived solely from the casein and yeast. The Ca/P ratios of 1: 1, 1: 2 and 1: 3 in diets 2, 3, and 4, respectively, were obtained by the addition of neutral sodium phosphate to the diets. The calcium and phosphorus content of each diet was verified by analysis.

The food intake of the 4 animals in each quadruplicate group was kept approximately the same from day to day and exactly equalized at 10-day intervals. At the end of a 70-day feeding period the animals were sacrificed and the jaw bones removed for examination of the teeth for the incidence and extent of caries. Examinations were made under a dissecting microscope and the teeth scored for caries according to the method described previously (Haldi and Wynn, '52).

Experiment II. This experiment was designed to obtain non-carious teeth from rats of the same inbred colony when fed the same diets for the same length of time as in the first experiment. The teeth of the desalivated animals in the first experiment became carious within the experimental period. If these teeth had been analyzed, the effect of diet on tooth composition could not have been determined with any degree of accuracy because extensive caries produces a loss of tooth substance and possible changes in the chemical composition of the remaining tooth substance. In some instances in the desalivated rats of the first experiment practically all the enamel was destroyed by the carious lesion.

The objective in the second experiment was realized by repeating the first experiment on animals from the same colony and under identical conditions with the exception that in the second experiment the rats were not desalivated. These rats

do not develop an appreciable amount of caries within 70 days on a cariogenic diet when they are not desalivated. At the end of this period in the second experiment, there were some minute brown areas in the fissures of a few of the teeth which were most likely due to the initiation of caries, but these areas were so infrequent and so small that they could not have had a measurable effect on the chemical composition of the teeth.

At the conclusion of the experiment the animals were sacrificed and the teeth removed, cleaned and dried. They were then ground to pass through a 100-mesh sieve and dried in a vacuum over P_2O_5. In order to obtain sufficient tooth substance for analysis the teeth of 4 animals on the same diet were pooled. The teeth of their litter mates on different diets were likewise pooled. Enamel was separated from dentine by the flotation method of Manly and Hodge ('39) as modified by Gilda ('51).

Analyses of the enamel and dentine for nitrogen, calcium, phosphorus, magnesium, fluoride and carbon dioxide were carried out by the following procedures: nitrogen by the micro-Kjeldahl method; calcium, Sobel, Rockenmacher and Kramer, '44; phosphorus, Fiske and Subbarow, '25; carbon dioxide, Van Slyke and Folch, '40 as modified by Sobel, Rockenmacher and Kramer, '44; magnesium, Young and Gill, '51; fluoride, Methods of Analysis, A.O.A.C. ('50) with titration as described by Williams, '46.

RESULTS

The number of carious lesions and the caries scores of rats on the 4 diets are given in table 1. The addition of phosphorus to the diet, thereby decreasing the Ca/P ratio from 1:0.5 to 1:1, resulted in a smaller number of lesions and a lower score. This effect was accentuated by further addition of phosphorus to give a Ca/P ratio of 1:2. Beyond this point neither the number of lesions nor the caries score were significantly affected by adding more phosphorus to the diet (Ca/P = 1:3) as shown in the statistical evaluation in table 1.

The results of the chemical analyses are given in tables 2 and 3. Inspection of these tables shows that varying the Ca/P ratio in the 4 diets had no significant effect on the nitrogen, magnesium, carbon dioxide, fluoride, calcium or phosphorus content of either the enamel or the dentine. The Ca/P ratios

TABLE 1

Dental caries in albino rats fed the same high sugar diet but with different Ca/P ratios

NUMBER OF ANIMALS	DIET	Ca	P	Ca/P RATIO	AV. NUMBER OF CARIOUS LESIONS		AV. CARIES SCORE	
		%	%					
40	1	0.49	0.24	1:0.5	22	± 4.2	44	± 18
40	2	0.50	0.52	1:1.0	18	± 4.7	25	± 11
40	3	0.50	0.98	1:2.0	13	± 6.1	16	± 8
40	4	0.50	1.48	1:3.0	11	± 5.3	13	± 8

Statistical evaluation

DIET	NUMBER OF CARIOUS LESIONS	CRITICAL RATIO [1]	CARIES SCORE	CRITICAL RATIO [1]
1	22		44	
		4.0		5.5
2	18		25	
		4.1		8.4
3	13		16	
		1.6		1.7
4	11		13	

[1] The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. When the critical ratio is: less than 2.0, the difference between the means is considered to be statistically insignificant; 2.0 to 2.9, the difference is of borderline significance; 3.0 or more, the difference is highly significant. (Dunning, J. M., 1950, J. Dent. Res., *29:* 541.)

in the enamel and dentine likewise were not affected by changing the Ca/P ratio in the diet.

Incidently, it may be noted that the gain in weight of the animals on each of the 4 diets was the same. This was not unexpected in view of the fact that the food intake of each quadruplicate group was balanced.

TABLE 2

Chemical analysis of dried enamel of the molars of albino rats on diets with different Ca/P ratios[1]

Ca/P RATIO IN DIET	N	Mg	F	CO_2	Ca	P	Ca/P RATIO
	%	%	%	%	%	%	
1:0.5	0.21 ± 0.03	0.12 ± 0.02	0.007	2.41 ± 0.06	32.5 ± 0.2	16.3 ± 0.2	1.99 ± 0.02
1:1.0	0.19 ± 0.01	0.14 ± 0.03	0.007	2.44 ± 0.03	33.2 ± 0.2	16.7 ± 0.2	1.99 ± 0.02
1:2.0	0.17 ± 0.03	0.10 ± 0.04	0.007	2.41 ± 0.03	33.4 ± 0.3	16.5 ± 0.1	2.02 ± 0.01
1:3.0	0.17 ± 0.01	0.12 ± 0.03	0.007	2.28 ± 0.08	33.1 ± 0.3	17.1 ± 0.2	1.95 ± 0.02

[1] Each value in the table is an average of 4 pooled samples. Each pooled sample was obtained from the teeth of 4 animals. In view of the small number of samples the ± values are given for the average deviations instead of the standard deviations.

TABLE 3

Chemical analysis of dried dentin of the molars of albino rats on diets with different Ca/P ratios[1]

Ca/P RATIO IN DIET	N	Mg	F	CO_2	Ca	P	Ca/P RATIO
	%	%	%	%	%	%	
1:0.5	2.85 ± 0.10	0.29 ± 0.01	0.010	3.90 ± 0.12	25.9 ± 0.3	12.7 ± 0.1	2.04 ± 0.03
1:1.0	2.82 ± 0.09	0.28 ± 0.01	0.010	3.85 ± 0.14	26.7 ± 0.3	13.3 ± 0.2	2.01 ± 0.03
1:2.0	2.78 ± 0.08	0.28 ± 0.01	0.010	3.84 ± 0.16	26.3 ± 0.2	13.0 ± 0.2	2.02 ± 0.02
1:3.0	2.78 ± 0.04	0.30 ± 0.02	0.010	3.83 ± 0.23	26.8 ± 0.2	13.3 ± 0.1	2.02 ± 0.02

[1] Each value in the table is an average of 4 pooled samples. Each pooled sample was obtained from the teeth of 4 animals. In view of the small number of analyses the ± values are given for the average deviations instead of the standard deviations.

TABLE 2

Chemical analysis of dried enamel of the molars of albino rats on diets with different Ca/P ratios[1]

Ca/P RATIO IN DIET	N	Mg	F	CO_2	Ca	P	Ca/P RATIO
	%	%	%	%	%	%	
1:0.5	0.21 ± 0.03	0.12 ± 0.02	0.007	2.41 ± 0.06	32.5 ± 0.2	16.3 ± 0.2	1.99 ± 0.02
1:1.0	0.19 ± 0.01	0.14 ± 0.03	0.007	2.44 ± 0.03	33.2 ± 0.2	16.7 ± 0.2	1.99 ± 0.02
1:2.0	0.17 ± 0.03	0.10 ± 0.04	0.007	2.41 ± 0.03	33.4 ± 0.3	16.5 ± 0.1	2.02 ± 0.01
1:3.0	0.17 ± 0.01	0.12 ± 0.03	0.007	2.28 ± 0.08	33.1 ± 0.3	17.1 ± 0.2	1.95 ± 0.02

[1] Each value in the table is an average of 4 pooled samples. Each pooled sample was obtained from the teeth of 4 animals. In view of the small number of samples the ± values are given for the average deviations instead of the standard deviations.

TABLE 3

Chemical analysis of dried dentin of the molars of albino rats on diets with different Ca/P ratios[1]

Ca/P RATIO IN DIET	N	Mg	F	CO_2	Ca	P	Ca/P RATIO
	%	%	%	%	%	%	
1:0.5	2.85 ± 0.10	0.29 ± 0.01	0.010	3.90 ± 0.12	25.9 ± 0.3	12.7 ± 0.1	2.04 ± 0.03
1:1.0	2.82 ± 0.09	0.28 ± 0.01	0.010	3.85 ± 0.14	26.7 ± 0.3	13.3 ± 0.2	2.01 ± 0.03
1:2.0	2.78 ± 0.08	0.28 ± 0.01	0.010	3.84 ± 0.16	26.3 ± 0.2	13.0 ± 0.2	2.02 ± 0.02
1:3.0	2.78 ± 0.04	0.30 ± 0.02	0.010	3.83 ± 0.23	26.8 ± 0.2	13.3 ± 0.1	2.02 ± 0.02

[1] Each value in the table is an average of 4 pooled samples. Each pooled sample was obtained from the teeth of 4 animals. In view of the small number of analyses the ± values are given for the average deviations instead of the standard deviations.

DISCUSSION

It would appear from these experiments that if the chemical composition of fully erupted teeth can be modified by the mineral composition of the diet, any change of this nature may occur in the teeth must proceed very slowly. If there were any such modifiations in the composition of the teeth in these experiments, they were so extremely small that they could not be detected by ordinary micro-analytical methods.

Sobel and Shaw (Sobel, '52) found that a diet with a high (7.5:1) Ca/P ratio produced high carbonate teeth which were more susceptible to decay than low carbonate teeth. Our experiments show that varying the Ca/P ratio of the diet may modify its cariogenicity without having an effect on the composition of the teeth. It should be emphasized however, that the results of these experiments cannot be applied as a test to the hypothesis advanced by Sobel that resistance of teeth to caries may be affected by their chemical composition which in turn may be related to the diet fed during the period of tooth calcification. In his experiments feeding was begun before the teeth were fully calcified. In our experiments the animals were placed on the diets at 23 days of age, at which age, according to Mellanby ('39), the molar teeth of albino rats are practically completely calcified.

In view of the fact that in our experiments there was no difference in the composition of the teeth of the animals on the different diets, the mechanism accounting for the difference in the cariogenicity of these diets obviously was not the same as in the experiments of Sobel and Shaw. At present we are unable to offer any suggestions as to the nature of this mechanism.

The progressive reduction in the cariogenicity of the diet that occurred with a change in the Ca/P ratio from 1:0.5 to 1:1 to 1:2 could not have been due to differences in the calcium content of the diets for this was maintained at a constant level. It must therefore have been related either to the increase in phosphorus or to the change in the Ca/P ratio or a combination of both of these factors. This issue cannot be settled on

the data presently available but must await clarification by further experimental studies.

SUMMARY

Quadruplicate groups of litter mate albino rats were fed from weaning on cariogenic diets in which 4 different Ca/P ratios were tested; the ratio was changed by maintaining the calcium concentration at a constant level and varying the phosphorus.

In one experiment in which the animals were desalivated, there was a progressive decrease in the caries score as the Ca/P ratio was decreased from 1 : 0.5.to 1 : 2. Further decrease in the ratio to 1 : 3 had no effect on the incidence or extent of caries.

In a second experiment in which the animals were not desalivated in order to avoid the complications of caries over the same feeding period, the nitrogen, calcium, phosphorus, magnesium, fluoride and CO_2 content of the teeth was the same on each of the 4 diets.

The effect of varying the Ca/P ratios of the diet on its cariogenicity was, therefore, not related to changes in the composition of the teeth.

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REPRODUCTION AND LACTATION OF RATS FED GLYCERYL TRILAURATE-CONTAINING DIETS

LESLIE P. DRYDEN, PAUL F GLEIS AND ARTHUR M. HARTMAN

Dairy Husbandry Research Branch, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland

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Keane et al. ('51) reported that female rats fed a diet containing glyceryl trilaurate plus methyl linoleate as a fat source were unable to rear their young to weaning age. The females appeared to conceive normally and to suckle normally. None the less, these young died or were eaten by their mothers, generally within the first few days of life. This deficiency could be overcome by supplementing the diet with 2.5% of wheat germ oil. Corn oil at the same level was found to be partially effective. The authors concluded that wheat germ oil and natural fats such as corn oil contain a hitherto unidentified factor(s) (not identical with any of the known vitamins or essential fatty acids) which is necessary to permit the female rat to rear her young.

In connection with a study of the nutritive value of butterfat as compared to vegetable fats, an effort was made in this laboratory to produce a similar deficiency with the intention of comparing the relative effects of butterfat and vegetable fats in overcoming such a deficiency. The basal ration employed and the experimental procedures followed were similar to those employed by Keane et al. The results of these attempts at producing such a deficiency are given in the present paper.

EXPERIMENTAL PROCEDURE

The experimental animals used were female albino rats primarily of the Wistar strain but derived from a colony maintained in this laboratory for many years. They were reared by colony mothers which were transferred at parturition from the stock ration (see Hartman et al., '51, for composition) to one of the experimental rations as indicated below.

Two rations were used: ration 1 consisted of 63.4% sucrose, 25.0% alcohol-extracted casein (see Hartman et al., '51 for extraction procedure), 4.5% salts (Jones and Foster, '42)[1], 5.0% glyceryl trilaurate,[2] 1.7% methyl linoleate,[3] 0.05% fish liver oil [4] and 0.38% vitamins.[5] Ration 2 differed from ration 1 only in that a commercial "vitamin-free" casein [6] was used and the fish liver oil was replaced by synthetic vitamin A acetate [7] and calciferol.[8]

Fresh diets were made at approximately weekly intervals and stored in sealed Mason jars at 27°F. or below between feedings. Glass feeders were used and a complete change of food was made each week. The rations and distilled water were supplied ad libitum.

In experiment 1, the colony mothers with their young were placed at parturition on ration 1. The young were weaned at 21 days of age, reared to 77 days of age on ration 1 and then mated to stock males. These experimental females were con-

[1] Modified to contain the following additional ingredients (micrograms per gram of total salt mixture): $K_2Al_2(SO_4)_4 \cdot 24H_2O$, 92; NaF, 506.

[2] Obtained from El Dorado Oil Works, San Francisco, California.

[3] "Methyl linoleate-60%," Nutritional Biochemicals Corp., Cleveland, Ohio.

[4] Containing per gram, 65,000 I.U. of vitamin A and 13,000 I.U. of vitamin D.

[5] In milligrams per 100 gm of ration: thiamine-HCl, 1.60; riboflavin, 1.60; pyridoxine HCl, 1.60; Ca pantothenate, 10.00; choline Cl, 240.00; nicotinic acid, 10.00; inositol, 10.00; para-aminobenzoic acid, 60.00; biotin, 0.020; pteroylglutamic acid, 0.20; ascorbic acid, 10.00; alpha-tocopherol acetate, 20.00; 2-methyl-1,4-naphthoquinone, 0.50; vitamin B_{12}, 0.010. The pteroylglutamic acid was kindly supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., and the crystalline vitamin B_{12} by Merck and Co., Inc., Rahway, N. J.

[6] Labco Casein, The Borden Co., New York, N. Y.

[7] Myvax, Distillation Products Industries, Rochester, N. Y., potency: 1.5 million I.U./gm. Included in ration at rate of 3158 I.U. per 100 gm ration.

[8] 0.016 mg/100 gm ration.

TABLE 1

Reproduction and lactation of rats fed glyceryl trilaurate and crude methyl linoleate as the fat component of the diet

NO. OF LITTER	NO. OF MOTHERS	MATINGS RESULTING IN PREG-NANCIES	PREG-NANCIES RESULTING IN LITTERS	AV. NO. OF YOUNG FOUND AT BIRTH		AV. BIRTH WT. OF LIVING YOUNG	SURVIVAL OF YOUNG AT 3 WEEKS OF AGE [1]	AV. BODY WT. OF YOUNG AT 3 WEEKS OF AGE		AV. CHANGE IN WT. OF MOTHER DURING LACTATION
				Living	Dead			Females	Males	
		%	%			*gm*	%	*gm*	*gm*	*gm*
				Experiment 1; ration 1						
1	56	86	100	8.8	0.5	5.8	87	35	36	— 23
2	55	94	100	7.9	0.5	6.0	94	37	39	— 21
3	23	84	98	7.6	0.4	6.3	94	38	40	— 22
4	18	85	100	6.2	0.8	6.0	82	40	38	— 18
				Experiment 1; ration 2						
3	27	82	100	8.4	0.5	6.1	90	40	46	— 21
4	22	91	100	7.3	0.2	6.4	93	41	42	— 26
				Experiment 2; ration 2						
1	24	92	100	7.4	0.1	6.3	81	37	37	— 12
2	24	94	99	7.9	0.3	6.2	78	35	35	— 23

[1] Based on number found living at birth.

tinued on the same ration nntil two litters had been carried to weaning. Then they were divided into two groups, one placed on ration 2 and the other maintained on ration 1 and carried through two additional pregnancies and lactations. Experiment 2 was carried out similarly except that the animals were fed ration 2 throughout the experiment. These latter rats were carried through two litters on the experimental rations.

RESULTS AND DISCUSSION

The results of these experiments are shown in table 1. It is evident that they fail to confirm the findings of the Illinois workers. Survival of young to weaning ranged from 78 to 94% (stock colony average: 88%) as contrasted to 0% found at Illinois. There is no evidence of failure in any other aspect of reproduction or lactation.

The explanation for these divergent results is not clear. The rations used, especially ration 2, were very similar to those used at Illinois. The methyl linoleate concentrate used is admittedly a crude source of the essential fatty acids and could conceivably be a carrier of unidentified nutrients. However, it is the same commercial product used by Keane et al.[9] in the diets on which they obtained the deficiency. In growth experiments with chicks, a 60% linoleic acid concentrate similar to that used here has been reported to give inconsistent results (Carver, '54) and at times to be growth depressing (Carver and Johnson, '54). The growth-depressing results were attributed to the fact that the product was in a late stage of incipient rancidity when used. Thus different lots of this concentrate might vary not only in content of possible unidentified nutrients but also in the extent to which they might induce symptoms of toxicity. However, in the course of the present experiments, 4 different purchased lots of this product were used without any noticeable effect on the results. The Illinois workers have suggested [9] that variable results ob-

[9] Personal communications from Dr. B. Connor Johnson and Dr. M. Daniel Lane, University of Illinois.

tained in experiments of this type may possibly be explained on the basis of the glyceryl trilaurate used. The product used in the present work was obtained from the same commercial source but was a different manufactured lot. Other possible explanations for these conflicting results lie, of course, in the different strains of rats used and variation in intestinal synthesis of possible unidentified nutrients from one rat colony to another.

SUMMARY

Experiments were carried out with rats fed certain diets containing glyceryl trilaurate plus methyl linoleate as a fat source. With such diets, other workers have found a deficiency of an unidentified factor required by the female rat to rear her young successfully. No such deficiency was found in the present work.

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THE QUANTITATIVE AMINO ACID REQUIREMENTS OF YOUNG WOMEN

IV. PHENYLALANINE, WITH AND WITHOUT TYROSINE [1]

RUTH M. LEVERTON,[2] NORMA JOHNSON,[3] JOAN ELLISON, DONNA GESCHWENDER AND FLORENCE SCHMIDT [2]

Nebraska Agricultural Experiment Station, University of Nebraska, Lincoln

TWO FIGURES

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Phenylalanine has been classified as an essential amino acid by Rose et al. ('43, '51), who reported that it must be supplied preformed in the diet to maintain human subjects in nitrogen equilibrium. Based on a study of 6 young men, Rose et al. ('55) have proposed 1.1 gm of L-phenylalanine as a tentative minimum daily requirement for human males. These workers also reported that 28 men were maintained in positive nitrogen balance on daily intakes of 2.4 gm or less of DL-phenylalanine. The experimental diets of all of the subjects were devoid of tyrosine.

At the University of Nebraska studies have been made of the quantitative requirements of young women for 5 of the essential amino acids.[4] Studies of the threonine, valine and tryptophan requirements have been reported (Leverton et al., '56a, b, c); this paper will report the results of the study of phenylalanine requirement with and without tyrosine.

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[2] Present address: Division of Home Economics, Oklahoma A. & M. College, Stillwater.

[3] Present address: Spokane, Washington.

[4] Supported in part by a U. S. Department of Agriculture contract sponsored by the Human Nutrition Research Branch, Agricultural Research Service.

PROCEDURE

The plan of the study, the description of the techniques, and the composition of the semi-purified diet have been described in the first paper of this series by Leverton et al. ('56a). The procedure used to determine the requirement involved reducing the intake of phenylalanine stepwise until the subjects were in negative nitrogen balance and then increasing it until balance was re-established. During this phase of the study there was a generous amount of tyrosine in the diet. After information was secured on the phenylalanine requirement, the amount of tyrosine was varied.

Two groups of college girls served as subjects for these investigations. Group A included 7 subjects. Six were girls identified as subjects 51, 53, 55, 57, 58, 59, and were each studied on several different levels of phenylalanine for 30 to 35 consecutive days. The other subject, subject 66, was studied on only one level of intake.

Group C included subject 58 and 7 other subjects, identified as A through H, who were on the Test Mix ration for 6 or 7 days after they had been subjects for the study of another amino acid. This ration supplied the 5 amino acids under investigation in the least amounts which had been found capable of maintaining nitrogen equilibrium in all of the subjects previously studied.

The 7 subjects in group A plus three additional ones, subjects 61, 62 and 68, were used to study the effect of tyrosine on phenylalanine requirement. The tyrosine was first omitted from the diet at one or more levels of phenylalanine intake and then added in varying amounts.

The daily nitrogen intake of each subject was 9.5 gm; it did not vary more than 0.2 gm throughout the study. Glycine and diammonium citrate were the chief sources of nitrogen and supplied equal amounts by the ration. The amounts of the purified amino acids which were fed to groups A and C are shown in table 1.

The auxiliary foods used daily to make the semi-purified diet more acceptable included 100 gm of reconstituted frozen orange juice and 75 gm each of canned pineapple and canned peaches. Group C also had 25 gm of grape juice daily. Microbiological assay of these foods with *Leuconostoc mesenteroides* P-60 showed that their phenylalanine content ranged from 17 to 21 mg. In reporting the results, therefore, 20 mg is

TABLE 1

The purified amino acids fed in the study of phenylalanine requirement

AMINO ACID	GROUP A	GROUP C
	Amount equivalent to 20 gm egg protein	Amount in Test Mix
	gm/person/day	*gm/person/day*
L-Arginine·HCl	1.549	1.549
L-Histidine·HCl	0.519	0.519
L-Lysine·HCl	1.800	1.800
L-Tyrosine	0.900 [1]	0.900
L-Phenylalanine	1.260 [1]	0.200
L-Trytophan	0.300	0.150
L-Cystine	0.480	0.480
L-Methionine	0.820	0.820
L-Threonine	0.980	0.184 [2]
L-Leucine	1.840	0.600
DL-Isoleucine	3.200	3.200
L-Valine	1.460	0.550
Nitrogen content	1.910	1.583

[1] These amounts were varied during the studies of requirement.

[2] Subjects C and F needed 275 mg plus 30 mg in the auxiliary foods.

added to the intake of purified L-phenylalanine to denote the total intake. The foods were not assayed for tyrosine. The auxiliary foods for group C were assayed also for the other 4 amino acids being measured and found to supply daily approximately 30 mg of threonine, 100 mg of valine, 7 mg of tryptophan and 20 mg of leucine.

Throughout this report all of the phenylalanine intakes referred to are the L-form of the amino acid and the amounts discussed are on a daily basis.

RESULTS

The mean daily nitrogen balances of each subject studied on each level of phenylalanine with a generous amount of tyrosine (900 mg) and with no tyrosine are charted in figure 1.

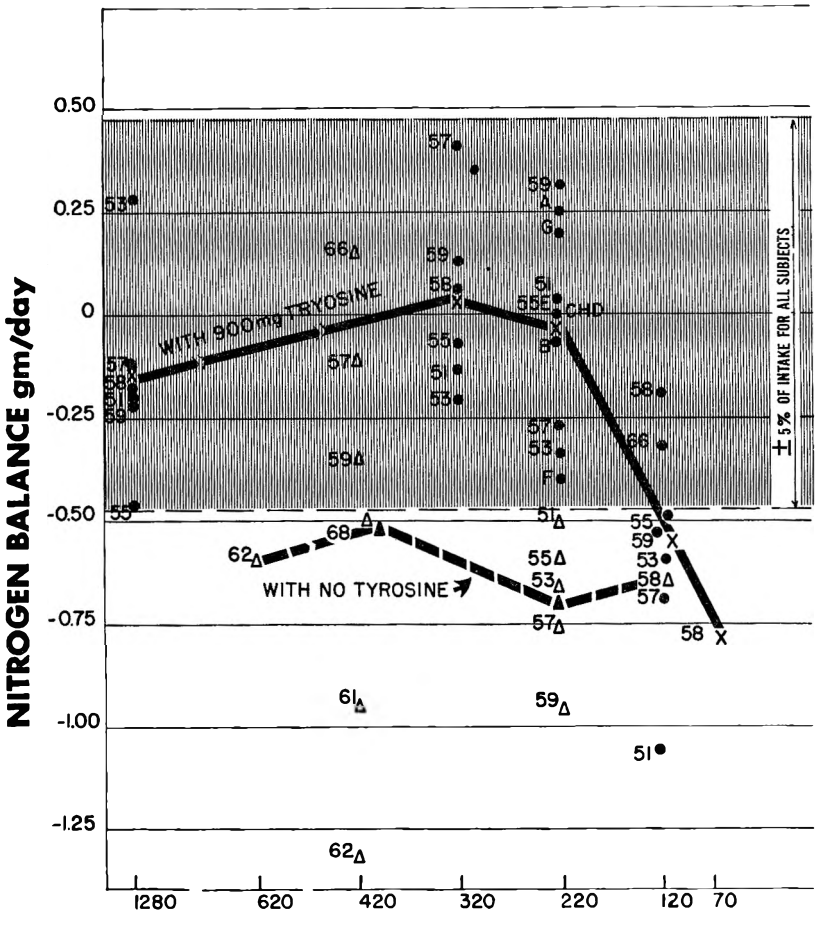

Fig. 1 Phenylalanine intake and nitrogen balance.

Each subject is identified by her code which appears beside her retention for each level on which she was studied.

Intake includes 20 mg/day of phenylalanine from auxiliary foods.

● represents subjects receiving 900 mg of tyrosine, mean = X.

△ represents subjects receiving no tyrosine, mean = ▲

The mean values for all subjects on each intake are also shown and connected with a line to emphasize the effect on nitrogen balance of decreasing the phenylalanine intake. The shaded portion indicates a zone of equilibrium or the area where the total nitrogen excretion is within 95 to 105% of

TABLE 2

Nitrogen balance of subjects on different levels of phenylalanine intake

LEVEL OF L-PHENYLALANINE [1]	NUMBER OF SUBJECTS ON EACH LEVEL	TOTAL NUMBER OF SUBJECT-DAYS	NITROGEN BALANCE				NUMBER OF SUBJECTS IN NEGATIVE BALANCE [3]
			Mean	S.D.[2]	Range		
					Low	High	
mg/day			*gm/day*		*gm/day*	*gm/day*	
With 900 mg tyrosine							
Group A							
1280	6	45	— 0.15	0.24	— 0.45	0.27	0
320	6	38	0.03	0.22	— 0.21	0.41	0
220	5	43	— 0.05	0.26	— 0.33	0.32	0
120	6	56	— 0.56	0.28	— 1.07	— 0.18	5
70	1	5	— 0.76	0.46	— 1.17	0.02	1
Group C Test Mix							
220	8	52	0.00	0.19	— 0.40	0.25	0
Groups A and C							
220	13	95	— 0.02	0.21	— 0.40	0.32	0
With no tyrosine							
620	1	5	— 0.59	0.34	— 1.00	— 0.17	1
420	6	38	— 0.52	0.54	— 1.31	0.16	3
220	5	34	— 0.69	0.17	— 0.94	— 0.50	5
120	1	7	— 0.63	0.46	— 1.53	— 0.13	1

[1] Includes 20 mg supplied by the auxiliary foods.

[2] Standard deviation.

[3] Nitrogen excretion more than 105% of the intake.

the intake. A nitrogen balance is not considered negative until the excretion is more than 105% of the intake.

The individual data in figure 1 for all of the subjects on each intake have been summarized in table 2 to show the mean daily nitrogen balance, standard deviation, range, total number of days the subjects were studied, and number of subjects in negative balance.

Phenylalanine levels with 900 mg of tyrosine

The 6 subjects with a generous intake of tyrosine remained in the zone of nitrogen equilibrium when the phenylalanine intake was reduced from 1280 to 320 mg daily. Five of these subjects were then given 220 mg daily and they remained in equilibrium. When their intake was decreased further to 120 mg of phenylalanine they went into negative nitrogen balance. Only subject 58 remained in balance on 120 mg, and she went into negative balance when her intake was reduced to 70 mg of phenylalanine. Subject 66 was in balance on an intake of 120 mg daily but was not studied on other levels with 900 mg of tyrosine.

The lowest phenylalanine intake (with 900 mg of tyrosine) which kept all of the subjects in group A in the zone of nitrogen equilibrium was 220 mg daily, 200 mg as the pure amino acid plus 20 mg from the auxiliary foods. These amounts, therefore, were included in the Test Mix ration. The 8 subjects in group C maintained nitrogen balance on these levels; none had a total nitrogen excretion which was more than 105% of the intake.

The difference between the mean nitrogen balance of -0.02 ± 0.21 gm on 220 mg of L-phenylalanine and -0.56 ± 0.28 gm on 120 mg is statistically significant at the 1% level of probability (by Student's t test, $t = 4.91$). Differences between the mean balances on 1280, 320 and 220 mg are not significant.

The smallest total daily intakes of phenylalanine (with 900 mg of tyrosine) which maintained the different subjects in nitrogen balance are shown in table 3. They are also expressed as mg/kg$^{3/4}$ actual body weight/day to show any differences in requirement which might be attributable to differences in the metabolic size of the subjects. Additional data presented include creatinine coefficients and caloric intakes. No relationships are apparent between the various items and the amount of phenylalanine required for nitrogen balance.

TABLE 3

Lowest intakes of phenylalanine used in this study which maintained nitrogen equilibrium [1]

DESCRIPTION	SUBJECT CODE	L-PHENYLALANINE INTAKE [2]		NO. DAYS	MEAN NITROGEN BALANCE	MEAN CREATININE COEFFICIENT	MEAN CALORIC INTAKE	AGE
		mg/day	*mg/kg$^{3/4}$/day*		*gm/day*		*cal/kg/day*	*yr.*
Group A								
Subjects who	51	220	11.5	10	0.03	21.5	44.9	22
were studied on	53	220	11.5	11	— 0.33	21.6	47.3	19
both higher and	55	220	11.5	5	0.00	24.0	48.6	21
lower intakes	57	220	10.4	12	— 0.27	22.3	39.9	21
than shown here	59	220	9.4	5	0.32	21.7	37.1	22
	58	120	5.6	13	— 0.18	19.1	36.0	20
	66 [3]	120	5.8	9	— 0.32	20.8	44.4	20
Group C Test Mix								
Subjects who	A	220	9.6	4	0.25	23.2	37.9	20
were studied only	B	220	10.3	7	— 0.06	21.6	42.7	22
on an intake of	C	220	9.2	7	0.00	21.6	36.8	19
220 mg	D	220	10.8	7	0.00	20.0	45.4	19
	E	220	10.6	7	0.02	22.7	45.2	20
	F	220	10.7	4	— 0.40	22.0	44.7	22
	G	220	9.8	7	0.20	22.9	41.3	22
	H	220	12.3	9	0.00	20.7	53.4	20

[1] Nitrogen excretion within 95%–105% of the intake.
[2] Accompanied by 900 mg tyrosine.
[3] This subject was studied on this level only.

None of the subjects showed or reported any symptoms, other than negative nitrogen balance, which might have suggested an inadequate intake of a dietary essential.

Phenylalanine levels with no tyrosine

Omitting the tyrosine at various levels of phenylalanine intake had a marked effect on nitrogen balance (table 2). When the tyrosine was withdrawn from the diets of 5 subjects who were receiving 220 mg of phenylalanine daily with 900 mg of tyrosine, the mean daily nitrogen balance dropped from -0.05 ± 0.26 gm to -0.69 ± 0.17 gm. The difference is significant at the 1% level of probability ($t = 4.61$). Subject 58, who had needed only 120 mg of phenylalanine with 900 mg of tyrosine for equilibrium, also went into negative balance when tyrosine was omitted from the mixture of purified amino acids fed.

The data in figure 1 for the subjects who were receiving no tyrosine show that:

1. When the phenylalanine intakes of subjects 57 and 59 were raised from 220 to 420 mg their nitrogen balances changed from negative to equilibrium. Subject 66 was also in equilibrium on an intake of 420 mg of phenylalanine.
2. Subjects 61, 62 and 68 were in negative balance on this intake of 420 mg of phenylalanine.
3. Subject 62 had the largest negative nitrogen balance on this intake of 420 mg of phenylalanine. Increasing her intake to 620 mg improved her balance but did not bring her into nitrogen equilibrium.

Phenylalanine levels with different levels of tyrosine

The next step was to vary the amount of tyrosine used with certain levels of phenylalanine. Phenylalanine intakes of 200 and 420 mg daily were used for this phase with tyrosine intakes of 200 and 450 mg.

The results are shown in figure 2 and summarized in table 4. For purposes of comparison figure 2 includes certain

values from figure 1 — the nitrogen balances of subjects on intakes of 900 mg of tyrosine and 0 mg of tyrosine with 220 and 420 mg of phenylalanine.

When subjects 51, 55, 59, 61 and 66 were given 220 mg of phenylalanine plus 200 mg of tyrosine, only subjects 55 and

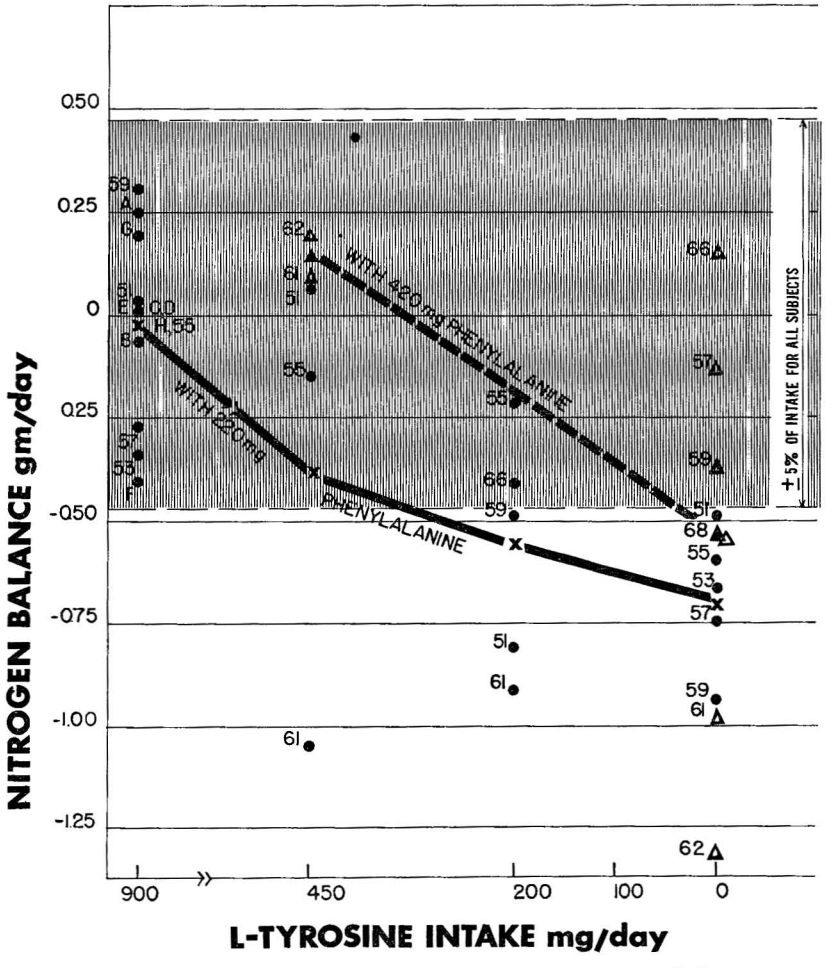

Fig. 2 Tyrosine and phenylalanine intake and nitrogen balance.

Each subject is identified by her code which appears beside her retention for each level on which she was studied.

● represents subjects on 220 mg of phenylalanine, mean = X.

△ represents subjects on 420 mg of phenylalanine, mean = ▲

66 had nitrogen balances in the equilibrium zone. When the intakes of subjects 51 and 61 were increased from 200 to 450 mg, and the phenylalanine intake kept at 220 mg, subject 51 came into equilibrium but subject 61 did not. With this level of tyrosine, subject 61 came into equilibrium when her intake of phenylalanine was raised from 220 to 420 mg.

The only significant difference between the nitrogen balances on different levels of tyrosine occurred when the phenylalanine intake was 220 mg. The mean nitrogen balance of -0.05 ± 0.26 gm when the tyrosine intake was 900 mg is significantly different from the mean balance of -0.56 ± 0.29 gm when the tyrosine intake was only 200 mg ($t = 4.50$ which is significant at the 1% level of probability).

TABLE 4

Nitrogen balance of subjects on different levels of tyrosine and phenylalanine intake

LEVEL OF L-PHENYLALANINE [1]	LEVEL OF L-TYROSINE	NO. OF SUBJECTS ON EACH LEVEL	TOTAL NO. OF SUBJECT-DAYS	NITROGEN BALANCE				NUMBER OF SUBJECTS IN NEGATIVE BALANCE [3]
				Mean	S.D.[2]	Range		
						Low	High	
mg/day				*gm/day*		*gm/day*	*gm/day*	
220	200	5	28	—0.56	0.29	—0.89	—0.21	3
420	450	2	10	0.12	0.08	0.06	0.18	0
220	450	3	15	—0.38	0.59	—1.05	0.06	1

[1] Includes 20 mg supplied by the auxiliary foods.

[2] Standard deviation.

[3] Nitrogen excretion more than 105% of the intake.

DISCUSSION

The amount of phenylalanine needed for nitrogen equilibrium by the subjects in this study ranged from 120 to 220 mg per day when 900 mg of tyrosine was also provided. On the basis of metabolic body size the phenylalanine intake ranged from 5.6 mg to 11.5 mg/kg$^{3/4}$/day for the subjects who were studied on several different intakes. This range included all but one of the phenylalanine intakes of the subjects on the Test Mix ration; at the 220 mg intake for subject H the value was 12.3 mg/kg$^{3/4}$/day.

The 13 subjects who were studied on a daily intake of 220 mg of phenylalanine with 900 mg of tyrosine remained in the zone of nitrogen equilibrium. On the basis of mean metabolic size this was a phenylalanine intake of 10.5 mg/kg$^{3/4}$/day. The 8 subjects on the Test Mix ration demonstrated the adequacy for nitrogen balance of 220 mg of phenylalanine (with 900 mg of tyrosine) when 4 other essential amino acids, threonine, valine, tryptophan and leucine, were present in similarly established minimum amounts.

Based on the results of this study an intake of 220 mg of L-phenylalanine daily is suggested as a tentative minimum requirement for young women for nitrogen balance when the diet also supplies 900 mg of L-tyrosine.

In order to compare this amount with the minimum requirement of 1.1 gm suggested by Rose et al. ('55) for men, it is necessary to allow for the phenylalanine replacement value of the tyrosine supplied in the present study. It has been reported that 70 to 75% of the phenylalanine requirement may be met with tyrosine (National Research Council, '53). On this basis the figure of 220 mg of phenylalanine in the present study would represent about 30% of what it would have been with no tyrosine. Therefore, the requirement of young women for phenylalanine without tyrosine might be approximately 800 mg, about three-fourths of the 1.1 gm of phenylalanine suggested for men.

The results of feeding the particular levels of phenylalanine and tyrosine used in this study showed no clear-cut quantitative relationships between the two amino acids. However, the sparing effect of tyrosine was apparent. Tyrosine had a favorable effect on nitrogen balance at all levels of phenylalanine intake studied, except at the lowest level of phenylalanine studied (120 mg) with and without tyrosine. Nitrogen balances of 4 of the 5 subjects on an intake of 450 mg of tyrosine with 220 mg and with 420 mg of phenylalanine were comparable to the nitrogen balances of subjects receiving 900 mg of tyrosine and 220 mg of phenylalanine. Further studies are needed especially using intakes within a range

of 220 to 620 mg of phenylalanine and 100 to 450 mg of tyrosine.

The range in daily phenylalanine intakes from diets of common foods has been reported by Mertz et al. ('52) as 0.9 to 3.7 gm, by Futrell et al. ('52) as 1.98 to 4.88 gm, and by Wharton et al. ('53) as approximately 2.5 to 2.7 gm. The patterns of amino acid consumed by people in different countries vary in phenylalanine content from 1.2 to 1.5 gm per 100 gm of protein (Allison, '53). Block and Bolling ('51) have calculated the average daily per capita consumption of the essential amino acids for the total population in the United States 1937 to 1941. The phenylalanine intake was 4.7 gm daily and the tyrosine intake was 3.9 gm.

SUMMARY AND CONCLUSIONS

Ten college girls served as subjects in a study of the requirement for phenylalanine with and without tyrosine. On a semi-purified diet the daily phenylalanine intake was reduced from 1280 to 320, to 220, to 120, and to 70 mg, while the tyrosine intake was kept constant at 900 mg daily. An intake of 220 mg of phenylalanine was the least amount which maintained all of the subjects in nitrogen equilibrium, defined as the condition in which nitrogen excretion remains within 95 to 105% of the intake. The effect of feeding other proportions of phenylalanine and tyrosine was studied but no clear-cut relationships were established. However, the sparing action of tyrosine on phenylalanine requirement was evident.

Another group of 8 subjects remained in the zone of nitrogen equilibrium when fed a Test Mix ration which included 220 mg of phenylalanine, 900 mg of tyrosine, and minimum amounts of threonine, valine, tryptophan and leucine found in this laboratory to be required by young women for maintaining nitrogen balance.

In the presence of 900 mg of tyrosine, 220 mg of phenylalanine is suggested as the tentative minimum daily requirement of young women.

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THE QUANTITATIVE AMINO ACID REQUIREMENTS OF YOUNG WOMEN

V. LEUCINE [1]

RUTH M. LEVERTON,[2] JOAN ELLISON, NORMA JOHNSON,[3]
JEAN PAZUR, FLORENCE SCHMIDT [2] AND
DONNA GESCHWENDER
Nebraska Agricultural Experiment Station,
University of Nebraska, Lincoln

ONE FIGURE

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Rose and co-workers have reported on both the qualitative and quantitative requirements of human subjects for leucine for the maintenance of nitrogen equilibrium (Rose et al., '43, '51; Rose, '49). A daily intake of 1.1 gm of leucine is suggested as a minimum daily requirement, and 2.2 gm was reported to be a satisfactory intake for 14 young men who were serving as subjects for studies of amino acids other than leucine (Rose, '52).

Studies have been made at the University of Nebraska of the requirement of young women for the essential amino acids threonine, valine, tryptophan, phenylalanine (Leverton et al., '56a, b, c, d), and leucine.[4] This paper will report the results of the work with leucine, the 5th and last amino acid which was studied.

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[2] Present address: Division of Home Economics, Oklahoma A. & M. College, Stillwater.

[3] Present address: Spokane, Washington.

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PROCEDURE

The method of study involved finding the least amount of leucine which would maintain nitrogen balance in the subjects. The plan of the study, the description of the techniques, and the detailed composition of the semi-purified diet are described in the first paper of this series by Leverton and co-workers ('56a).

Three groups of subjects were used in the study of leucine requirement. Group A included 8 college girls, identified as subjects 38, 61, 62, 63, 65, 67, 68 and 69, who were each studied on several different levels of leucine. A preliminary study was made with subject 38 about a year before the major study of leucine was initiated with the other 7 subjects in group A (Leverton et al., '56a, table 1). Group B included 4 subjects, 34, 36, 37, 41, who, at the end of the tryptophan study, were tested on an intake of 710 mg of L-leucine. This was the smallest amount of leucine studied which had kept subject 38 in equilibrium.

There were 8 subjects in group C, identified as A through H. They were used to check the adequacy of a Test Mix ration which included the 5 acids — leucine, threonine, valine, tryptophan and phenylalanine — in the least amounts which had been found to maintain nitrogen equilibrium in the subjects previously studied. Subject A had been identified as subject 58 during the phenylalanine study and subjects B through H had been subjects 61, 62, 63, 65, 67, 68 and 69 respectively during the leucine study. This was the only one of the 5 studies reported in this series in which the group of subjects used for studying the requirement of an amino acid was also used in a subsequent study on the Test Mix ration.

The semi-purified diet was made up of cornstarch, sugar, butter and corn oil, agar flakes, lemon juice, mineral and vitamin supplements and amino acids (Leverton et al., '56a). The following canned fruits which were low in nitrogen were added daily to make the diet more acceptable: 100 gm of

frozen reconstituted orange juice, 75 gm of pineapple, 75 gm of peaches, and 25 gm of grape juice

The composition of the purified amino acid mixture given to each group of subjects is shown in table 1. Aliquots of foods and chemicals were analyzed for nitrogen before being fed, and the total daily nitrogen intake was brought up to 9.5 gm with glycine and diammonium citrate. The variation in the daily nitrogen intake for each subject did not exceed 0.2 gm throughout the study.

TABLE 1

The purified amino acids fed in the study of leucine requirement

AMINO ACID	GROUPS A AND B	GROUP C
	Amount equivalent to 20 gm egg protein	Amount in Test Mix
	gm/person/day	*gm/person/day*
L-Arginine·HCl	1.549	1.549
L-Histidine·HCl	0.519	0.519
L-Lysine·HCl	1.800	1.800
L-Tyrosine	0.900	0.900
L-Phenylalanine	1.260	0.200
L-Tryptophan	0.300	0.150
L-Cystine	0.480	0.480
L-Methionine	0.820	0.820
L-Threonine	0.980	0.184 [2]
L-Leucine	1.840 [1]	0.600 [3]
DL-Isoleucine	3.200	3.200
L-Valine	1.460	0.550
Nitrogen content	1.910	1.583

[1] The amount of leucine was varied during the study of requirement.

[2] Subjects C and F required 275 mg plus 30 mg from the auxiliary foods.

[3] Subject A received 690 mg.

The foods were assayed microbiologically with *Leuconostoc mesenteroides* P-60 for leucine and found to contain from 17 to 20 mg of L-leucine daily. Therefore, in reporting the levels of leucine intake, 20 mg has been added to the amounts of purified L-leucine fed. All of the leucine intakes referred to in this report denote the L-isomer and the amounts always refer to the daily intake. The foods used for group C were assayed also for the other 4 amino acids being measured

and found to contain approximately 30 mg of threonine, 100 mg of valine, 7 mg of tryptophan, and 20 mg of phenylalanine.

RESULTS

The nitrogen balance of each subject on each level of leucine intake is shown in figure 1. The shaded portion marks the zone of equilibrium or the area in which the total nitrogen

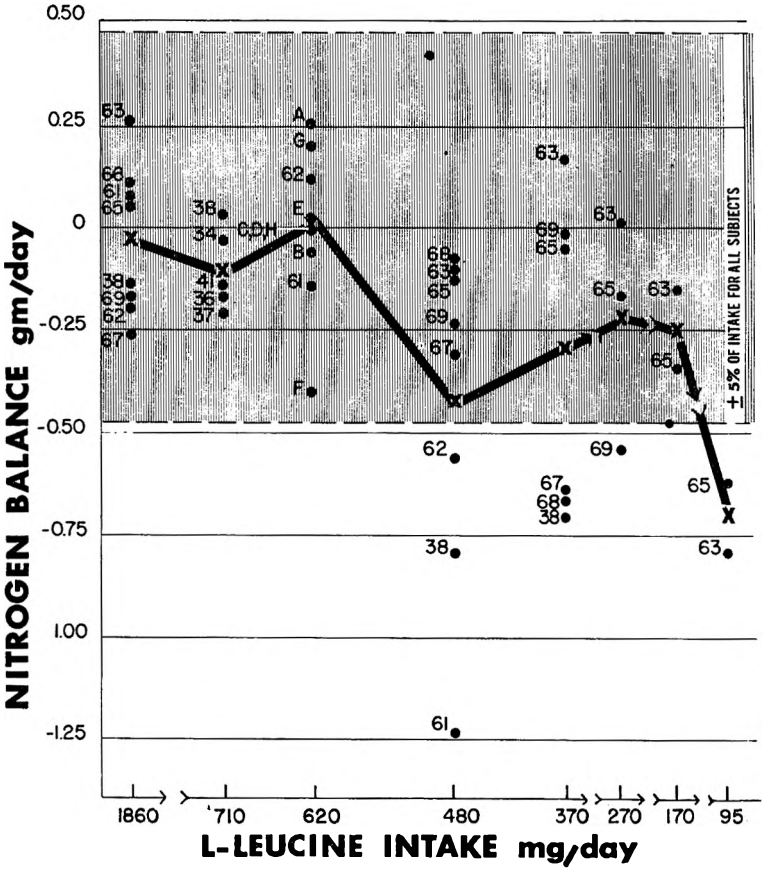

Fig. 1 Leucine intake and nitrogen balance, 13 subjects for 334 subject-days.

Each subject is identified by her code which appears beside her retention for each level on which she was studied.

X = mean retention of all subjects on each level of intake.

Intake includes 20 mg/day supplied by auxiliary foods.

excretion is within 95 to 105% of the nitrogen intake. The criterion which has been used for judging negative nitrogen balance in these studies is a total excretion which is more than 105% of the nitrogen intake. In figure 1 the mean balances of all the subjects on each intake of leucine are connected with a solid line. The median values of the balances are not indicated but inspection shows that they are almost identical to the means.

There was great variation among the nitrogen balances of the subjects when the daily intake of leucine was below 620 mg. For subjects 63, 65 and 69 especially, much lower intakes were needed to produce negative balance than for the other subjects. The relation between the extent of the negative balances and the level of intake, therefore, was not as consistent among the different subjects for leucine as it was for the other amino acids which have been studied.

The nitrogen balances of the individual subjects are summarized in table 2 which records the levels of leucine fed, the mean, standard deviation, and range of the nitrogen balances, and the number of subjects in negative balance on each level of leucine. The length of time that each subject was on each level of intake was similar but not identical. It depended on the variability of her nitrogen balance, the extent to which the balance was negative, and the appearance of any symptoms of ill health. The total number of days that all of the subjects were on each level of intake is given in table 2 and referred to as "subject-days."

On intakes of 1860, 710 and 620 mg of leucine all of the subjects had nitrogen balances within the zone of equilibrium. When the intake was reduced to 480 mg, differences in the subjects' responses appeared; three of the 8 subjects were in negative balance. Reducing the intake further to 370 mg of leucine caused two more subjects to go into negative balance, but subjects 63, 65 and 69 remained in nitrogen equilibrium. Then the leucine intakes were dropped to 270 mg, but only subject 69 went into negative balance. Subjects 63 and 65 remained in balance on 170 mg of leucine, and not

until their intakes were reduced to 95 mg of leucine were their nitrogen balances distinctly negative.

Student's t test showed that the only significant difference between the nitrogen balances on different intakes of leucine was between the mean balance of the subjects on 480 mg and those on 620 and 710 mg. This difference is significant at the 1% level of probability ($t = 3.64$).

TABLE 2

Nitrogen balance of subjects on different levels of leucine intake

LEVEL OF L-LEUCINE [1]	NUMBER OF SUBJECTS ON EACH LEVEL	TOTAL NUMBER OF SUBJECT-DAYS	NITROGEN BALANCE				NUMBER OF SUBJECTS IN NEGATIVE BALANCE [3]
			Mean	S.D.[2]	Range		
					Low	High	
mg/day			*gm/day*		*gm/day*	*gm/day*	
Group A							
1860	8	57	— 0.03	0.18	— 0.26	0.25	0
710	1	8	0.03	0.40	— 0.27	0.46	0
620	2	12	— 0.01	0.52	— 0.14	0.12	0
480	8	88	— 0.43	0.41	— 1.35	— 0.08	3
370	6	43	— 0.32	0.39	— 0.70	0.17	3
270	3	21	— 0.23	0.28	— 0.54	0.01	1
170	2	11	— 0.25	0.13	— 0.34	— 0.15	0
95	2	11	— 0.71	0.12	— 0.79	— 0.62	2
Group B							
710	4	31	— 0.14	0.08	— 0.21	— 0.03	0
Group C Test Mix							
620	8	52	0.00	0.19	— 0.40	0.25	0
Groups A and C							
620	10	64	— 0.02	0.10	— 0.40	0.25	0

[1] Includes 20 mg supplied by the auxiliary foods.

[2] Standard deviation.

[3] Nitrogen excretion more than 105% of the intake.

In table 3 the least amount of leucine used in this study which would maintain each subject in nitrogen equilibrium is expressed in terms of metabolic size ($kg^{3/4}$ actual body weight) and shown with the nitrogen balance, creatinine coefficient, caloric intake, and age of each subject. The amount which was needed ranged from 8.2 to 31.0 mg of leucine per

TABLE 3

Lowest intakes of leucine used in this study which maintained nitrogen equilibrium [1]

DESCRIPTION	SUBJECT CODE	L-LEUCINE INTAKE		NO. DAYS	MEAN NITROGEN BALANCE	MEAN CREATININE COEFFICIENT	MEAN CALORIC INTAKE	AGE
		mg/day	*mg/kg$^{3/4}$/day*		*gm/day*		*cal/kg/day*	*yr.*
Group A								
Subjects who	38	710	31.0	8	0.03	19.3	37.9	20
were studied on	61	620	29.1	7	— 0.14	22.8	42.7	22
both higher and	62	620	25.9	5	0.12	21.2	36.8	19
lower intakes	67	480	23.4	15	— 0.31	22.3	44.7	22
than shown here	68	480	21.3	12	— 0.08	23.7	41.3	22
	69	370	20.7	10	— 0.01	22.4	53.4	20
	63	170	8.3	5	— 0.15	20.7	45.4	19
	65	170	8.2	6	— 0.34	17.9	45.2	22
Group B								
Subjects who	34	710	30.9	9	— 0.03	21.2	40.1	21
were studied on	36	710	32.1	7	— 0.16	19.9	39.8	23
an intake of	37	710	32.1	7	— 0.21	19.9	46.3	19
710 mg	41	710	34.3	8	— 0.15	21.2	42.6	23
Group C Test Mix								
Subjects who	A	710	31.0	4	0.25	23.2	37.9	20
were studied on	B (61)[2]	620	29.1	7	— 0.06	21.6	42.7	22
an intake of	C (62)	620	25.9	7	0.00	21.6	36.8	19
620 mg or 710 mg	D (63)	620	30.4	7	0.00	20.0	45.4	19
with 4 other	E (65)	620	29.9	7	0.02	22.7	45.2	22
essential amino	F (67)	620	30.2	4	— 0.40	22.0	44.7	22
acids present in	G (68)	620	27.5	7	0.20	22.9	41.3	22
minimum amounts	H (69)	620	34.6	9	0.00	20.7	53.4	20

[1] Nitrogen excretion within 95 to 105% of the intake.
[2] Number of each subject when she was on the leucine study.

$kg^{3/4}$/day for the subjects who were studied on several levels of intake. Subject 38 who needed 31 mg/$kg^{3/4}$ or 710 mg per day was not studied on an intake of 620 mg of leucine as were the other subjects in group A; then she was in negative balance on 480 mg of leucine daily or 21 mg/$kg^{3/4}$. For the subjects who were used for checking (group B) and for the subjects on the Test Mix ration (group C) the intakes ranged from 26.4 to 34.6 mg of leucine per $kg^{3/4}$/day. However, subjects D through H on the Test Mix ration had maintained nitrogen equilibrium on lower intakes of leucine when they were in group A. No relationship is apparent between the leucine requirement of these subjects and the creatinine coefficients. In group A the three subjects, 69, 63, 65, who needed the least amount of leucine for nitrogen balance had the highest caloric intake for the maintenance of body weight.

When subjects 62, 65, 67 and 68 were in negative nitrogen balance they had a few symptoms of gastrointestinal distress which may have been attributable to an inadequate intake of a dietary essential. In one case a meal was regurgitated.

DISCUSSION

The variability among subjects in their requirement for leucine was greater than for any of the other 4 amino acids which have been studied. The least amount of these amino acids which was required by different subjects for nitrogen equilibrium varied from 103 to 305 mg for L-threonine, 465 to 650 mg for L-valine, 82 to 157 mg for L-tryptophan, and 120 to 220 mg for L-phenylalanine (with 900 mg tyrosine). For leucine, however, the variation was from 170 to 710 mg. Rose, Warner and Haines ('51) have reported that the nitrogen balances of two young men responded slowly and unevenly to both the removal and restoration of leucine in a purified diet.

Suggesting a tentative minimum requirement for leucine is more difficult than for the other amino acids studied. At the time subject 38 was used for the pilot study, the great variability among subjects in their responses to different

levels of leucine had not been encountered. Therefore, when subject 38 was in nitrogen equilibrium on a leucine intake of 710 mg and in distinct negative nitrogen balance on 480 mg, an intake of 710 mg was used for group B. When the other 7 subjects in group A were studied the great variability was evident, i.e., 5 subjects were in nitrogen balance on intakes varying from 170 to 480 mg. For the Test Mix ration it seemed unnecessary to use a leucine level as high as 710 mg; instead a level of 620 mg was used because this was the highest requirement found for the group A subjects studied after subject 38. The Test Mix ration proved adequate for all of the subjects. The question is whether subject 38 and group B would have remained in nitrogen equilibrium on a leucine intake of 620 mg. Their individual nitrogen balances are similar to the balances of subjects 61 and 62 on 620 mg, who, like subject 38, were in negative balance when the intake was decreased to 480 mg of leucine daily. Considering results presented here, and until results of further studies are available, the authors are suggesting 620 mg of L-leucine as a tentative minimum requirement for maintaining nitrogen balance in young women.

The minimum daily requirement of 1.1 gm of L-leucine proposed by Rose ('49) is well above this figure of 620 mg suggested here for young women. In terms of metabolic size, 1.1 gm of L-leucine is 45 mg/kg$^{3/4}$/day for a 70-kg man, as compared with 30 mg/kg$^{3/4}$/day, the mean value for the young women on the present study.

Reports in the literature indicate that generous amounts of leucine can be supplied from food. Futrell et al. ('52) found that the self-chosen diets of 4 women contained from 3.28 to 7.35 gm of leucine daily. The self-chosen diets of 18 women studied by Mertz et al. ('52) assayed from 1.3 to 7.8 gm of leucine daily. Diets of common foods planned by Wharton et al. ('53) furnished approximately 4.4 to 4.9 gm of leucine daily. In Allison's ('53) presentation of Block's figures for calculations of the amino acids consumed by hu-

mans on different diets, the amounts of leucine vary from 7.5 to 9.2 gm per 100 gm of protein.

This is the last report of the studies in this series on the amino acid requirements of young women. Much more work needs to be done with subjects similar to the ones studied here and with subjects in different age groups. Knowledge of requirements is fundamental to evaluating food supplies, planning production, and implementing supplementation where it is needed. From results available at present it appears that the amounts of threonine, valine, tryptophan, phenylalanine, and leucine in the food supplies available to the people in this country are not likely to be limiting factors in nitrogen utilization.

SUMMARY AND CONCLUSIONS

The leucine requirement has been studied, with 12 young women serving as subjects on a semi-purified diet. Daily leucine intakes of 1860, 710, 620, 480, 370, 270, 170 and 95 mg were fed, and nitrogen balances determined. Not all of the subjects were studied on all of these levels.

The amount of leucine needed by the different subjects for nitrogen equilibrium (excretion within 95 to 105% of the intake) varied from 170 to 710 mg daily. The 710 mg intake was for only one subject who required more than 460 mg of leucine but who was not studied on an intake of 620 mg. When 620 mg was included in a Test Mix ration with minimum amounts of 4 other essential amino acids, threonine, valine, tryptophan and phenylalanine, all 8 of the subjects fed this ration were in nitrogen equilibrium.

The figure of 620 mg of L-leucine has been suggested as a tentative minimum daily requirement for young women for nitrogen equilibrium.

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DIETARY DEPRIVATION OF RIBOFLAVIN AND BLOOD RIBOFLAVIN LEVELS IN MAN [1]

OTTO A. BESSEY, M. K. HORWITT AND RUTH H. LOVE

Department of Biochemistry and Nutrition, The University of Texas-Medical Branch, Galveston, and Biochemical Research Laboratory, Elgin State Hospital, Elgin, Illinois

ONE FIGURE

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The development of practical microchemical analytical methods for riboflavin, flavin mononucleotide (FMN) and flavin dinucleotide (FAD) and the observation that the concentrations of some of these substances in the red blood cells, white blood cells and plasma of experimental animals vary with the dietary intake of riboflavin, made it feasible to consider further the use of blood analytical methods for the evaluation of nutritive status in man, with respect to this dietary essential (Burch et al., '48). This paper reports the results of blood riboflavin measurements on two groups of men (total of 18 subjects) maintained for 9 to 16 months on well controlled, restricted levels of riboflavin intake (0.5 to 0.55 mg of riboflavin daily), compared with two control groups (total of 29 subjects) receiving liberal riboflavin intakes (2.4 to 3.55 mg daily). It has been found that the total riboflavin content of the red blood cells decreases significantly and consistently between the second and 6th week after riboflavin restriction, and therefore, this analysis can serve as a practical means of evaluating riboflavin nutritive status. Although the plasma riboflavin level also decreases as a result of ribo-

[1] This study was supported in part by a grant from The National Vitamin Foundation.

flavin restriction, plasma analysis was found to be neither as convenient nor as reliable as red blood cell analysis, as an index of riboflavin status.

EXPERIMENTAL AND RESULTS

The blood riboflavin measurements were made as a part of two rather comprehensive studies conducted at Elgin State Hospital:[2] the first, from February 1947 to September 1948; the second, from September 1950 to October 1951.

Study 1

In the first study, one group of 10 subjects (group A) received a diet adequate in all other known respects, but containing only 0.55 mg of riboflavin per day during the 16 months previous to the date of the blood analyses. All 10 of these subjects showed some clinical manifestations of ariboflavinosis and three of them showed severe symptoms. The second group (group B) of 6 subjects received the same basal diet as those of group A, plus a total of 2.55 mg of riboflavin per day during the first 14 months, which was raised to 3.55 mg per day during the two months just preceding the analyses. The choice and care of subjects, the extent of dietary control, and reports of other laboratory and clinical findings may be found elsewhere (Horwitt et al., '49a, b, '50; Hills et al., '51). All blood riboflavin analyses were done in quadruplicate on postabsorptive specimens, by the methods previously described (Burch et al., '48).

The results of the analyses are summarized in table 1. It can be seen that (1) the total riboflavin content of red blood cells is strikingly and consistently lower in the riboflavin-restricted group (group A) as compared with that of subjects receiving a liberal riboflavin intake (group B); (2) the white blood cell levels of riboflavin are not significantly different in the two groups; (3) the free plus FMN riboflavin

[2] Various aspects of these studies are reported in the cited literature under the designation of Elgin State Hospital Projects 2 and 3 respectively.

of plasma is usually significantly lower in the restricted group. However, this last parameter may be disturbingly anomalous in certain individuals, as indicated by the two values (subjects 7 and 14) obtained in this study which were outliers.

TABLE 1

Summary of analyses of riboflavin content of blood in groups of men maintained on controlled riboflavin intakes

All values are averages of quadruplicate analyses in μg/100 ml of blood

SUBJECT NUMBER	RED CELL TOTAL RIBOFLAVIN	WHITE CELL TOTAL RIBOFLAVIN	PLASMA		
			Free + FMN [1] riboflavin	FAD [2] riboflavin	Total riboflavin
	Group A (Riboflavin intake 0.55 mg/day)				
1	11.1	252	0.62	1.37	1.99
3	12.4	194	0.04	2.62	2.66
4	11.4	204	0.24	1.86	2.10
6	11.5	220	0.34	2.24	2.58
7	12.7	218	1.43 [3]	2.91	4.34
8	13.1	190	0.28	2.50	2.78
9	11.7	162	0.22	1.82	2.04
10	10.0	140	0.59	2.16	2.75
11	10.9	169	0.12	2.25	2.37
12	11.9	174	0.04	1.95	1.99
Average ± S.D.	11.7 ± 0.9	192 ± 33	0.28 ± 0.18	2.17 ± 0.20	2.36 ± 0.35
Range	10.0 – 13.1	140 – 252	0.04 – 0.62	1.37 – 2.91	1.99 – 2.78
	Group B (Riboflavin intake 2.55–3.55 mg/day)				
2	27.6	245	0.72	2.52	3.24
5	22.9	245	0.74	2.24	2.98
13	20.6	216	0.68	2.21	2.89
14	20.6	185	1.87 [3]	2.26	4.13 [3]
15	20.2	207	0.81	2.72	3.53
16	21.8	175	0.61	1.89	2.50
Average ± S.D.	22.3 ± 2.8	212 ± 29	0.71 ± 0.074	2.31 ± 0.29	3.03 ± 0.39
Range	20.2 – 27.6	175 – 245	0.61 – 0.81	1.89 – 2.72	2.50 – 3.53

[1] Flavin mononucleotide.

[2] Flavin adenine dinucleotide.

[3] Values excluded from calculations of average, S.D. $\left(\sqrt{\frac{\Sigma x^2}{N-1}}\right)$, and range. Shown to be outliers by method of Dixon (*Annals of Mathematical Statistics, 21:* 488 [1950]).

(4) The plasma FAD riboflavin levels are not significantly changed by the restriction; (5) the total riboflavin analyses may also yield anomalous values, as would be expected, since the free plus FMN riboflavin concentration is a part of this total. In any event, since more than two-thirds of the total riboflavin in plasma comes from FAD riboflavin which, in turn, does not change appreciably with intake, total plasma riboflavin would appear to be not a very sensitive index of riboflavin intake.

It will be noted that there was one subject in each group in which the plasma free plus FMN riboflavin was strikingly out of line. It has been established that neither analytical error nor hemolysis of the specimens was responsible for these high values. It seems reasonably certain that these individuals did in fact have such high values, for reasons which are not as yet clear. We have since observed such anomalous plasma values in a number of other subjects. It is interesting that, in all instances, it has been only the free plus FMN riboflavin values of the plasma that are anomalous. The riboflavin contents of the red blood cells and the white blood cells in these subjects were similar to others in the group.

Study 2

The second study was designed primarily to investigate niacin requirements; however, in addition, it provided an opportunity by which further information on the riboflavin intake versus blood levels could be obtained. Four groups of 7 to 8 subjects each received a basal diet that was designed to be low in niacin (approximately 5.8 mg/day), tryptophan (265 mg/day), and riboflavin (0.4 mg/day) but which was made adequate in other known respects by the addition of a vitamin mixture and other essentials. Group I received the basal diet + 0.1 mg of riboflavin (total riboflavin = 0.5 mg/day); group II, basal diet + 2.0 mg of riboflavin (total riboflavin = 2.4 mg/day); group III, basal diet + 2.0 mg of riboflavin + 10 mg of niacin (total riboflavin = 2.4 mg/day);

group IV, basal diet + 2 mg of riboflavin + 100 mg of tryptophan (total riboflavin = 2.4 mg/day). A 5th group (group V), one receiving the regular hospital diet (total riboflavin approximately 1.6 mg/day), was included for analysis near the termination of the study. Blood specimens for analysis were collected before the subjects were placed on their respective dietary regimens and at intervals during the ensuing 9 months, after which time the 8 subjects in group I (restricted group) were divided into two groups, one of which (group Ia) received a total of 1.3 mg of riboflavin daily for 71 days, after which a total of 2.4 mg daily was given for the next 14 days; the other group (group Ib) received a total of 2.4 mg of riboflavin daily until the termination of the study (total of 85 days). The choice and care of subjects, extent of dietary control, and reports of other laboratory and clinical findings may be found elsewhere.[3] Five of the 8 subjects in group I had some clinical manifestations of ariboflavinosis, 4 of whom had relatively severe symptoms.

Since the first study had indicated that the red blood cell was the blood phase of choice for evaluating riboflavin nutritive status, only red blood cell analyses were done in this second study. The analyses were made in quadruplicate on postabsorptive specimens, by methods previously described (Burch et al., '48).

The numerical results of all analyses (averages of quaduplicates) are summarized in table 2, and the mean for each group at each of the periods of the analyses (A, B, C, D, etc.) is graphically presented in figure 1. It will be noted (1) that at the beginning of the study (period A) all groups are similar in red blood cell riboflavin content; (2) that within 45 days (period C) of the time of restriction of group I to 0.5 mg of riboflavin daily, the riboflavin content of the red blood cells of the individuals of this group is significantly lower than that in groups II, III, and IV who were receiving 2.4 mg of riboflavin daily, and that this difference slowly increased during the ensuing months of the study; (3) that

[3] Horwitt et al., unpublished data.

TABLE 2

Summary of total riboflavin content of red blood cells in groups of men maintained on controlled riboflavin intakes

All values are averages of quadruplicate analyses in μg/100 ml of blood

DAYS ON DIET	0	16	44	72	128	178	247	282	351
DATE OF ANALYSIS	A 9/27/50	B 11/22/50	C 12/20/50	D 1/17/51	E 3/14/51	F 5/3/51	G 7/11/51	H 8/15/51	I 10/23/51
				Group I (Riboflavin intake 0.5 mg/day)					
Subject 1	18.7	18.6	15.3	16.6	10.4	12.9	10.0	13.7 [1]	15.3 [2]
3	20.6	20.3	16.3	18.4	13.7	14.7	13.2	16.6 [1]	20.0 [2]
4	24.7	23.8	17.1	16.6	14.8	13.6	13.7	17.8 [1]	20.9 [2]
5	20.6	18.8	16.8	16.5	16.1	16.8	14.3	20.1 [3]	21.4 [3]
6	21.8	19.4	13.8	15.3	15.6	10.6	11.4	18.9 [3]	22.3 [3]
7	21.6	16.7	13.2	13.8	12.5	11.0	10.4	18.4 [3]	18.5 [3]
8	19.6	15.9	11.9	12.2	10.1	14.4	7.0	14.9 [3]	18.5 [3]
33	...	21.7	...	16.5	13.1	13.9	15.0	15.0 [1]	16.9 [2]
Average	21.1	19.3	14.9	15.7	13.3	13.5	11.9	15.8 [1] 18.1 [3]	18.2 [2] 20.2 [3]
S.E.	± 0.72	± 0.91	± 0.74	± 0.71	± 0.79	± 0.71	± 0.95	± 0.90 [1] ± 1.1 [3]	± 1.4 [2] ± 0.98 [3]
				Group II (Riboflavin intake 2.4 mg/day)					
Subject 9	18.2	20.5	21.7	23.1	22.9	22.7	20.1	20.6	19.5
10	17.9	22.7	19.3	26.6	24.0	23.1	21.3	24.9	20.2
11	23.3	22.5	24.6	29.0	24.8	23.3	26.3	28.4	23.6
12	23.9	21.7	22.7	28.0	21.0	21.5	23.5	26.1	23.1
13	19.8	20.3	20.7	24.4	26.5	23.9	23.9	26.3	22.9
14	19.4	18.1	19.7	20.4	20.5	19.7	...	22.9	17.2
15	16.2	18.4	...	20.3	19.8	20.6	21.3	22.3	19.1
24	20.0	17.9	17.2	20.3	15.0	18.3	17.7	17.4	15.8
Average	19.8	20.3	21.6	24.0	21.8	21.6	22.1	23.6	20.2
S.E.	± 0.93	± 0.69	± 0.92	± 1.3	± 1.3	± 0.70	± 1.1	± 0.85	± 1.0
				Group III (Riboflavin intake 2.4 mg/day)					
Subject 16	14.4	17.5	16.3	20.9	18.4	20.0	19.8	19.[illegible]	17.8
17	22.6	20.3	21.0	20.6	21.4	22.8	27.8	24.4	22.1
18	18.4	17.9	17.6	18.4	19.4	20.5	20.4	23.8	19.7
19	21.9	19.3	18.5	21.7	18.8	21.9	20.7	21.0	20.3

	21	21.8	16.5	15.3	19.6	12.7	18.5	15.7	19.2	17.2
	22	25.0	19.0	22.2	26.5	26.5	22.2	19.0	25.0	22.4
	23	19.3	19.6	17.8	21.2	17.8	19.4	19.9	18.9	13.0
Average		20.5	19.7	18.4	21.3	19.3	20.8	20.5	21.9	18.9
S.E.		± 1.3	± 0.50	± 0.93	± 0.96	± 1.6	± 0.60	± 1.4	± 0.98	± 1.2
				Group IV (Riboflavin intake 2.4 mg/day)						
Subject	25	21.0	21.2	17.5	18.6	19.3	21.5	23.3	20.7	22.2
	26	19.8	20.5	19.4	17.0	20.6	21.5	23.5	...	...
	27	20.7	21.5	12.7	18.3	20.5	21.4	25.2	20.4	20.7
	28	18.3	19.3	21.9	15.2	18.6	16.9	19.8	19.6	16.2
	29	23.3	22.0	22.8	23.9	25.6	25.2	26.0	25.9	25.0
	31	20.0	17.7	18.0	21.6	21.8	18.9	21.9	20.2	18.4
	32	17.0	17.2	16.2	19.8	21.6	20.0	21.7	19.9	...
	34	...	14.3	16.4	18.8	20.2	16.4	23.5	21.1	17.8
Average		20.0	19.2	18.1	19.2	21.3	20.2	23.1	21.1	20.0
S.E.		± 0.75	± 0.94	± 1.2	± 0.95	± 0.75	± 1.0	± 0.70	± 0.82	± 1.2
				Group V (Riboflavin intake 1.6 mg/day)						
Subject	2	21.1	...	17.2	...	..	..	19.6	20.3	17.8
	20	22.2	20.4	...	...	..	..	..	..	..
	30	16.9	...	...	...	..	..	17.3	19.0	16.8
	35	...	...	...	...	..	..	26.1	21.6	20.1
	36	...	...	...	...	..	..	18.1	18.2	18.8
	37	...	...	...	...	..	..	16.6	20.3	15.1
	38	...	...	...	...	..	..	18.8	18.0	14.2
	39	...	...	...	...	..	..	18.0	17.6	17.0
	40	...	...	...	...	..	..	21.3	21.0	18.8
	41	...	...	...	...	..	..	..	21.6	20.0
Average		20.1	20.4	17.2	...	..	.	19.5	19.7	17.6
S.E.		± 1.6	...	...	...	..	..	± 1.1	± 0.52	± 0.68

[1] 7/30/51 — 1.3 mg total riboflavin/day (Group Ia).
[2] 10/ 9/51 — 2.4 mg total riboflavin/day (Group Ia).
[3] 7/30/51 — 2.4 mg total riboflavin/day (Group Ib).

S.E. $= \sqrt{\frac{\Sigma x^2}{N(N-1)}}$.

groups II, III, and IV do not differ significantly from each other or with time; (4) that group V (hospital diet) is not different from groups II, III, and IV; (5) that the individuals in group Ib are completely realimented with riboflavin after receiving 2.4 mg of riboflavin for 85 days, a total of 204 mg (one subject, no. 5, appeared to be realimented after receiving

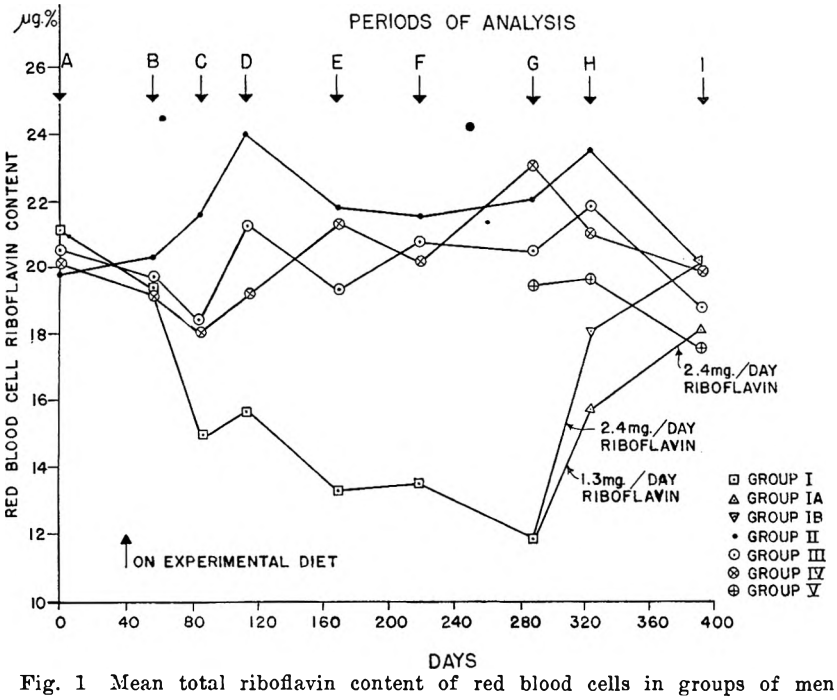

Fig. 1 Mean total riboflavin content of red blood cells in groups of men maintained on controlled riboflavin intakes. Group I, 0.5 mg riboflavin daily; groups II, III and IV, 2.4 mg riboflavin daily; group V, 1.6 mg riboflavin daily. Eight subjects in each group.

a total of only 36 mg of riboflavin over a 15-day period), while some of those in group Ia may not be completely realimented after receiving a total of 126 mg of riboflavin over a period of 85 days (subjects no. 1 and no. 33 had red blood cell riboflavin contents of only 15.3 and 16.2 μg/100 ml, respectively). However, both the number of subjects and the number of analyses are inadequate to determine whether or not this represents great variability in the requirements

for realimentation from individual to individual. It seems probable that the size of the dose of riboflavin is the primary limiting factor in these cases and not time, since rats which have been severely deficient for weeks can be realimented in a few hours, if sufficient riboflavin is given.

Statistical treatment of data [4]

Experimental error. Analytical error estimates are based on 142 samples of 4 determinations each. The estimated population standard deviation ($\bar{c}$) = 1.24 μg % and the standard error of the mean ($c_{\bar{x}}$) of quadruplicate determinations = 0.62 μg %. In terms of confidence limits, it is expected that 95% of the time the true riboflavin content of a specimen will not differ from the indicated mean of 4 determinations more than 1.22 μg % ($1.96\, c_{\bar{x}} = 1.96 \frac{\bar{\sigma}}{\sqrt{4}}$). Since previous estimates [5] indicate a 95% confidence limit of ± 0.2 μg % for the mean of quadruplicate riboflavin determinations on a single filtrate, it seems evident that a large part of the variability of the analyses results from the sampling of red blood cells.

Analysis of variance by groups and periods. Analysis of variance was performed in three distinct parts: (1) groups I to IV in periods C, F, and G; (2) groups I to IV in periods A and I; (3) groups I to V in period I.

Because of missing samples and the unequal number of replicate determinations, the analyses are based on the means of the actual number of determinations in each sample. The experimental error is thus eliminated from the analysis of variance. The missing items were supplied by the "missing plot" method (Snedecor, '46a) and the analysis of variance follows the method described by Snedecor ('46b).

Since the variability between determinations was eliminated by the use of the means, the subjects within each group are

[4] Prepared by M. C. Westbrook and J. A. Scott, Research Assistant and Professor of Statistics, respectively, Department of Preventive Medicine, The University of Texas-Medical Branch, Galveston.

[5] Bessey, unpublished data.

considered replicates and the term "error" refers to the variability between subjects treated alike. Total interaction was found to be significant at the 1% level as indicated in the "F" column of table 3. The total group effect was significant at the 1% level when tested against the total interaction, but the total period effect was not significant when so tested. As expected, the mean of group I for periods C, F, and G differed from that of the other groups; therefore, the total group effect and the total interaction were subdivided as shown in table 3. Both parts of the interaction were

TABLE 3

Analysis of variance of groups I to IV in periods C, F, and G

SOURCE	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
Groups	(3)	(881.11)	(293.70)	9.97 [1]
I vs other groups	1	838.98	838.98	23.49 [2]
Difference in II, III and IV	2	42.13	21.06	0.80
Periods	2	26.52	13.26	0.45
Interaction (G × P)	(6)	(176.69)	(29.45)	5.28 [1]
I vs other groups	2	71.45	35.72	6.40 [1]
Difference in II, III and IV	4	105.24	26.31	4.72 [1]
Error (subjects treated alike)	78	435.43	5.58	

[1] 1% level of significance.
[2] 5% level of significance.

found to be significant at the 1% level. When the subdivisions of the group effect were tested with their respective interactions, it was found that "I vs. other groups" was significant at the 5% level, while there was no significant difference between the other groups.

Thus, the principal interpretation is that groups II, III, and IV do not differ significantly from each other in periods C, F, and G, but group I shows a significantly lower level of riboflavin than the other groups during these periods.

When tests similar to those described for table 3 were applied to table 4, no significant differences were found for groups, periods, or interaction. In other words, the ribo-

flavin levels do not differ significantly either between groups at the beginning of the experiment (period A), between groups at the end of the experiment (period I), or between periods A and I.

No significant differences were found between groups I to V in period I (table 5), showing that group V which was on a hospital diet, does not differ significantly in riboflavin level from the groups on the special diets.

TABLE 4

Analysis of variance of groups I to IV in periods A and I

SOURCE	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
Groups	3	1.33	0.44	0.06
Periods	1	9.48	9.48	1.25
Interaction (G × P)	3	22.79	7.60	1.02
Error	51	380.67	7.46	

TABLE 5

Analysis of variance of groups I to V in period I

SOURCE	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
Groups	4	35.47	8.87	1.14
Error	33	256.72	7.78	

DISCUSSION AND CONCLUSIONS

It would appear from the above data and other recent observations that the red blood cell riboflavin content and the available microanalytical methods possess characteristics which make analysis of this phase of the blood a useful method for evaluating dietary intake with respect to riboflavin in man. The changes which occur upon restriction of dietary intake are sufficiently sensitive and uniform to provide a method of practical reliability not only for small groups of individuals, such as might be of interest in a dietary survey, but also for single individuals. Although further study will be required to determine what influence unusual physiological stresses or pathological processes may have on this parameter,

aside from an influence on requirements, the uniformity of response by the number of subjects over the period of time included in this study indicates that the major factor that influences riboflavin content of red blood cells is dietary intake.

It seems reasonable to conclude from present experience that a red blood cell riboflavin content of 20 μg/100 ml or more indicates an adequate intake. These levels occur in most individuals at intakes of 1.5 to 2.5 mg/day. Levels of intake above this, *i.e.*, 10 mg/day, raise the red blood cell level of riboflavin only temporarily for a few hours, after which it returns to the 20 to 25 mg/100 ml level. The conclusion that red blood cell levels of 20 μg and above are adequate is based, of course, on the assumption that an intake sufficient to "saturate" the red blood cell is all the body can hold and, therefore, surely adequate or more with respect to nutrition. It also seems reasonably safe to conclude that if the riboflavin content of the red blood cells falls below 15 μg/100 ml, this is an indication that the intake is approximately 0.5 mg/day and is good presumptive evidence that the individual needs more riboflavin in his dietary intake. In this study, all subjects, with one exception, who received such restricted intakes had red blood cell riboflavin levels below 15 μg/100 ml and most of them, as previously noted, developed clinical signs and symptoms of deficiency. Red blood cell riboflavin levels in the 15 to 20 μg/100 ml range are more difficult to interpret and, therefore, the background and philosophy of the interpreter will become an important factor in the meaning of such an analysis. Those who feel that if there is any question about the adequacy of intake of a dietary essential, it is wise to increase it, if conditions permit, will likely make such a recommendation in these cases, as a matter of insurance. Those who whould like more definite proof that individuals in this category are not "saturated" or are actually handicapped in health, will probably not be so concerned. If the truth were known, this middle group of subjects probably contains both individuals who would be helped by increased

riboflavin intake and those who would not. Present methods and information are simply inadequate to make the discrimination. However, it should be noted that growing experimental animals continue to show growth increments with increased riboflavin intakes nearly to the point of red blood cell riboflavin "saturation."[6]

The data that have been presented indicate that the level of free plus FMN riboflavin of the plasma also generally reflects the dietary intake. However, the use of this analysis as an index of riboflavin nutritive status is complicated by several factors, the most disturbing of which is the presence of numerous individuals in both adequately nourished and deficient groups who have plasma riboflavin levels two to 5 times the average. There appear to be conditions, as yet undefined but apparently not related to dietary intake, which lead to those "outlier" values. It has been shown in experimental animals that the action of some of the hormones leads to greatly increased levels of riboflavin in plasma (Common et al., '47). One also wonders whether plasma riboflavin might not increase in conditions of negative nitrogen balance, a situation which is known to lead to increased riboflavin excretion. In any event, at present, the use of plasma riboflavin values as a means of evaluating nutritive status involves uncertainties due to the possible effects of factors, other than dietary intake, of considerable relative magnitude. Also, although plasma analyses can be done with satisfactory precision, these may require more care to avoid accidental errors, due to the small quantities of riboflavin involved.

It is of interest that in the present study the level of FAD riboflavin in plasma was not decreased significantly in spite of a degree of dietary riboflavin restriction sufficient to produce clinical evidence of deficiency. Mann et al. ('52) found a striking decrease in this parameter in the growing Cebus monkey kept on a diet almost devoid of riboflavin. The severely restricted growing rat also shows such changes.[7] However,

[6] See footnote 5.

[7] See footnote 5.

with a lesser degree of riboflavin restriction in the rat and also, as in this case, in man, the plasma FAD riboflavin appears not to be significantly affected. Apparently, the stress of growth is a factor since the plasma FAD riboflavin of mature rats decreases but little even with a degree of deficiency that produces striking skin lesions. It appears that changes in plasma FAD riboflavin are an indication of severe deficiency under conditions in which growth is a stress factor.

In a like manner, the riboflavin content of the white blood cells did not change appreciably under the conditions of this study. Severe dietary restriction of riboflavin in the growing rat leads to a decrease in the riboflavin content of the white blood cells. Also, it should be noted that the riboflavin content of the white blood cells of two infants maintained on restricted riboflavin intake was lowered (Snyderman et al., '49). However, in these infants, as with adult man, the riboflavin content of the red blood cells was the most sensitive and convenient index of riboflavin intake. The analysis of riboflavin in white blood cells, while feasible, is technically more difficult than in red blood cells and subject to considerable potential sampling error.

Since the riboflavin content of whole blood will vary with the proportion of red blood cells to plasma, as well as with the nutritive level of the individual, such analyses are inadequate unless accompanied by a hematocrit measurement and proper corrections. This procedure is not recommended since it involves additional sources of error.

Horwitt et al. ('50) have reported in detail values for the urinary excretion of riboflavin in some of the subjects included in this report. Urinary excretion of riboflavin drops to approximately 50 μg/day within a few days of restriction to 0.55 mg of riboflavin per day and then slowly declines to about half this value during the following few months. It is clear that riboflavin excretion reflects current intake and that such measurements are, therefore, useful in evaluating nutritive status. Under some circumstances, this may be the method of choice rather than that of red blood cell analysis.

However, it should be noted that the amount of riboflavin excreted in the urine may be temporarily and misleadingly increased by the consumption of a small amount of a riboflavin-rich item of food, *e.g.,* milk. The excretion of such low levels is also significantly increased by a negative nitrogen balance. Red blood cell analyses are not affected by such temporary conditions but rather reflect the average of recent riboflavin intake. The method of choice will depend, of course, upon the circumstances of a particular project.

Questions naturally arise as to what factors other than dietary intake may affect the riboflavin content of the red blood cells and whether one finds in the population a significant number of individuals with red blood cell levels of riboflavin which indicate a possible need for more dietary attention in this respect. Analyses of red blood cells for total riboflavin have been done on 145 patients, selected at random in the John Sealy Hospital (Galveston) pediatric clinic, and on 142 adult patients from the population of the same hospital. Of the 145 children, 37 had red blood cell riboflavin levels in the 15 to 20 μg/100 ml zone and 21 were below 15 μg/100 ml, one of these being below 10 μg/100 ml. Among the adults, there were 41 in the 15 to 20 μg/100 ml zone and 13 below 15 μg/100 ml, three of which were below 10 μg/100 ml. There appears to be no significant correlation between the analyses and the medical conditions involved. It is not known whether these low values are due to low riboflavin intake per se or to other factors. However, these groups do provide a population with a significant number of low red blood cell riboflavin values for further study as to cause.

SUMMARY

The total riboflavin in red blood cells, white blood cells and plasma, and the free plus FMN riboflavin and FAD riboflavin in plasma were determined on one group of 10 men who had been maintained for 16 months on a well controlled dietary intake of 0.55 mg of riboflavin/day. Similar measurements were made on a control group of 6 men who

had received 2.55 to 3.55 mg of riboflavin/day. The riboflavin content of the red blood cells was 10.0 to 13.1 μg/100 ml in the restricted group and 20.2 to 27.6 in the control group. Although the free and FMN riboflavin of plasma was in general decreased in the restricted group, there were some striking exceptions. The plasma FAD riboflavin and the total white blood cell riboflavin were not significantly changed.

Analyses of riboflavin in red blood cells were done on a group of 8 men before and periodically during a 9-month maintenance on a riboflavin intake of 0.5 mg/day. Similar analyses were done on 4 control groups receiving 1.6 to 2.4 mg of riboflavin daily. The riboflavin content of the red blood cells became significantly lower in the restricted group within 45 days of the restriction and slowly decreased further during the ensuing months.

It is concluded that the red blood cell content of riboflavin is a reasonably sensitive and practical index for evaluating nutritive status with respect to this dietary essential and that available microchemical methods are well adapted for this measurement.

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THE ABSORPTION OF PLANT STEROLS AND THEIR EFFECT ON SERUM AND LIVER STEROL LEVELS

LEON SWELL, T. A. BOITER, HENRY FIELD, JR. AND C. R. TREADWELL [1]
General Medical Research, Veterans Administration Center, Martinsburg, W. Va., and The Department of Biochemistry, School of Medicine, George Washington University, Washington, D. C.

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In an earlier report (Swell et al., '54a) it was shown that soybean sterols incorporated into a high-cholesterol diet inhibit the development of hypercholesterolemia in rats. Soybean sterols were also found to be esterified *in vitro* by pancreatic cholesterol esterase under the same conditions as cholesterol, namely, in the presence of bile salts and a fatty acid source. It was postulated that soybean sterols inhibit the absorption of cholesterol by competing with cholesterol for the enzyme and its cofactors. Sterol specificity studies (Swell et al., '54b) demonstrated that pancreatic cholesterol esterase synthesized and hydrolyzed long-chain fatty acid esters of β-sitosterol, mixed sitosterols, dihydrocholesterol, ergosterol, and stigmasterol. It has been shown that cholesterol is esterified during absorption (Siperstein et al., '52) and that the esterification is probably catalyzed by pancreatic cholesterol esterase (Swell et al., '50; Hernandez et al., '55). In view of the finding that the same enzyme catalyzed the esterification of the plant sterols, it seemed possible that the plant sterols could be absorbed through the same mechanism as cholesterol.

The belief that plant sterols are not absorbed is based on the classical studies of Schoenheimer ('31) who fed rabbits,

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rats, and cats large amounts of sitosterol and observed that the total sterol in their bodies did not increase as it did if small amounts of cholesterol were given. Moreover, no sitosterol was found in the serum, tissues and lymph and all was recovered unchanged in the feces. Recent studies by Gould and co-workers (Gould, '54; Gould et al., '55) using the more sensitive method of feeding tritium-labeled sterols have indicated that dihydrocholesterol, mixed sitosterols, and β-sitosterol are absorbed, although not as well as cholesterol. The rate of disappearance from blood of the labeled plant sterols was much faster than for labeled cholesterol, suggesting the possibility that sitosterol does not accumulate in the body because of rapid excretion or degradation. The feeding of dihydrocholesterol led to an increase in liver sterols which suggested partial replacement of liver cholesterol by dihydrocholesterol; sitosterol had no effect on the liver sterol level. Gould ('54, '55) followed only the activity of the total sterol fraction and did not separate plant sterols from cholesterol. Ivy et al. ('54) employed the balance technique and fed dihydrocholesterol or soya sterols with oleic acid. The apparent absorption of dihydrocholesterol and soya sterols was 34.7 and 35.2%, respectively. Also, an increase was observed in the sterol level of plasma as determined colorimetrically. While the above-mentioned investigators have presented strong evidence that plant sterols are absorbed, these sterols have not been demonstrated in serum or liver following absorption.

If the blood cholesterol-lowering effect of dietary plant sterols is due to competition between dietary plant sterols and cholesterol (dietary and endogenous) for the cholesterol esterase system, then, assuming the plant sterols are not absorbed, a maximum lowering should occur on a cholesterol-free diet where the competition would involve only endogenous cholesterol. On the other hand, if plant sterols are absorbed, a cholesterol-free diet should allow increased absorption of plant sterols due to decreased competition by cholesterol. The purposes of the present investigation were to study the ab-

sorption of plant sterols on cholesterol-free diets, to determine the conditions favoring their absorption, the effect of their absorption on the serum and liver sterol levels, and their possible fate in the body.

METHODS AND MATERIALS

Animals and diets. Rats (male and female) weighing approximately 150 gm were used. The animals were housed in individual metabolism cages arranged for quantitative collection of feces and were allowed free access to water. The diets

TABLE 1

Feeding regimen on experimental diets

GROUP [1]	FINAL BODY WEIGHT	DIETARY ADDITIONS		
		Soybean sterol	Oleic acid	S. taurocholate
	gm	%	%	%
X-1	163 ± 6	0	0	0
X-2	195 ± 12	0	25	0
X-3	153 ± 8	0	0	1
X-4	173 ± 10	2	0	0
X-5	151 ± 13	2	0	1
X-6	201 ± 13	2	25	0
X-7	199 ± 21	0	25	1
X-8	192 ± 18	2	25	1
X-9	159 ± 11	0	5	0

[1] Seven rats per group; length of feeding period 21 days.

in slight excess of the daily requirement were weighed and placed in food cups. The diet remaining the following day was weighed and the food intake calculated by difference. The fat-free diets after being weighed were made into a paste with water to prevent spilling. Sterol intake was calculated from the data for food intake. The rats were divided into 9 groups of 7 each. Each group received a basal synthetic diet to which was added in various combination, 5 or 25% of oleic acid, 2% of soybean sterols, and 1% sodium taurocholate. The feeding regimen is shown in table 1. The basal diet had the following composition: 20% casein, 24% corn starch, 24% glucose, 2% roughage 5% Hubbell-Mendel-Wakeman salt mixture ('37),

and adequate amounts of the crystalline vitamins. The remaining 25% of the diet was oleic acid or in those diets containing no fat or 5% oleic acid, the difference was made up by additional carbohydrate. The soybean sterols and sodium taurocholate were substituted at the expense of the carbohydrate. The sterol content of the diets was checked by gravimetric analysis (Gardner et al., '38). All animals received the diets for 21 days. At the end of the experiment, the animals were anesthetized with ether, blood was obtained from the abdominal aorta, the liver removed, blotted free of blood, and weighed.

Feces. Seven-day quantitative collection of feces for each rat were dried at 70°C. to constant weight, pulverized to a fine powder, and extracted 4 times with boiling 2:1 chloroform-methanol. The pooled extracts were washed with water and made to volume. All of the lipids were found to be extracted by this procedure. Total lipids were determined by taking an aliquot of the extract and drying to constant weight. Total sterols were determined gravimetrically by saponifying the lipids with 10% alcoholic KOH, extracting the non-saponifiable lipids with ether, precipitating the sterols with digitonin, and weighing the digitonide (Gardner et al., '38).

Liver. The livers were homogenized in the Potter-Elvehjem apparatus with saline and made to volume. Total lipids were determined by extracting an aliquot of the homogenate with 2:1 chloroform-methanol, washing the extract with water, and drying the residue to constant weight. Free and total cholesterol was determined on each homogenate by the method of Sperry and Webb ('50). The homogenates were pooled in each group, the lipids saponified with 10% alcoholic KOH, and the non-saponifiable material extracted with ether. Total sterols were determined gravimetrically by weighing the digitonide.

Serum. Free and total cholesterol was determined on the serum (Sperry and Webb, '50). The sera of each group were pooled and total lipids and gravimetric sterols determined as for liver.

Chromatography. The sterol digitonides obtained from liver, serum, and feces were cleaved with cold pyridine, the digitonin precipitated with ether, and the sterols isolated. The sterols were chromatographed on Whatman no. 1 filter paper strips impregnated with paraffin, as described by Kodicek and Ashby ('54). The solvent system for separating plant sterols (sitosterols and stigmasterol) from cholesterol contained the following: ethylene-glycolmonoethyl ether 35: n-propanol 10: methanol 30: water 25. Complete separation of the plant sterols from the other digitonin precipitable sterols (cholesterol, 7-dehydrocholesterol, and dihydrocholesterol) in serum and liver could be achieved since sitosterol and stigmasterol did not migrate and remained at the origin. When the isolated serum and liver sterols of rats fed a sterol-free diet were chromatographed, all of the sterols were found to migrate. Ascending chromatograms (20 to 50 μg) were run for 16 to 17 hours at 34°C. The papers were dried at 90°C. for 4½ minutes, dipped into a freshly prepared saturated solution of $SbCl_3$ in chloroform, and heated for an additional 4 minutes at 90°C. The sterols appeared as rose-colored spots and as little as 2.5 μg of sterol could be detected; dihydrocholesterol was not detected since it did not give a color with the reagent. Thus, 2.5 μg of plant sterol could be detected in a total of 50 μg of sterol, or 5% of plant sterols in a mixture of plant sterol and cholesterol.

RESULTS

Sterol absorption. The sterol intake and fecal excretion of the rats on the different diets is shown in table 2. The sterol excretion data represent the average of two 7-day fecal periods per rat. Apparent sterol absorption was calculated by taking the respective control group excretion into account. The plant sterols were virtually all recovered in the feces of group X-4 which received the basal diet with 2% of soybean sterols. Group X-5, fed a similar diet with 1% of bile salts added, absorbed 25 mg/day or 15.7% of the plant sterols that were fed. This diet was fat free. Group X-6, fed the basal diet with

TABLE 2

The absorption of plant sterols and their effect on serum sterol levels

GROUP	STEROL INTAKE AND EXCRETION				SERUM STEROL AND LIPIDS				
	Sterol intake [1]	Sterol excretion [1]	Apparent sterol absorbed [2]	Apparent sterol absorption [2]	Serum sterols, colorimetric			Serum sterols, gravimetric	Non-sterol lipids
					Free	Ester [3]	Total	Total	
	mg/day	*mg/day*	*mg/day*	*%*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
X-1	0	5 ± 1			13.7	41.0	54.7 ± 9.7	60.0	174
X-2	0	6 ± 1			15.0	51.6	66.6 ± 5.0	67.1	180
X-3	0	6 ± 1			23.1	75.3	98.4 ± 31.0	92.0	156
X-4	154	156 ± 7	3 ± 3	1.9 ± 1.9	17.5	46.1	63.6 ± 14.8	62.0	177
X-5	161	142 ± 12	25 ± 14	15.7 ± 8.3	17.4	44.7	62.1 ± 5.1	68.0	203
X-6	170	152 ± 10	24 ± 10	15.5 ± 5.8	18.9	67.0	85.9 ± 9.9	87.1	337
X-7	0	6 ± 1			22.6	70.9	93.5 ± 14.7	89.1	259
X-8	175	141 ± 11	40 ± 7	22.9 ± 4.6	26.4	77.1	103.5 ± 13.4	104.4	245
X-9	0	5 ± 1			15.3	48.1	63.4 ± 8.4	63.2	184

[1] Represents average of two 7-day fecal periods per rat, sterols were determined gravimetrically.

[2] Taking control excretion into account.

[3] Expressed as free cholesterol.

25% of oleic acid, absorbed 24 mg/day or 15.5% of the amount fed. Group X-8, fed 25% oleic acid and 1% of bile salts, absorbed the greatest amount of plant sterol, 40 mg/day or 22.9% of the soybean sterols fed.

The control groups excreted approximately the same amount of sterols. A slight increase in fecal sterols was noted when fat, bile salts, or both were added to the diet.

Serum. The serum sterol and lipid levels are shown in table 2. In the groups not receiving soybean sterols, the addition of bile salts or oleic acid and bile salts (X-3 and X-7) to the basal diet increased the ester and total serum cholesterol level approximately 60% over the control group (X-1). The addition of 5 or 25% oleic acid to the basal diet had virtually no effect on the serum cholesterol level. Groups X-4 and X-5, which received 2% of soybean sterols, 2% of soybean sterols and 1% of bile salts, respectively, did not exhibit any changes in their serum cholesterol. Groups X-6 and X-8, which received 2% of soybean sterols in their diets with either 25% of oleic acid or 25% oleic acid and 1% bile salts, exhibited increases in their serum sterol level. These levels were higher than the corresponding groups not receiving plant sterol in their diet. Group X-8 had the highest serum sterol level (104.4 mg %) of all of the groups.

The non-sterol lipids were slightly higher in the groups receiving oleic acid in their diet. The gravimetric sterols were in close agreement with the colorimetric determinations.

Liver. The liver sterol and lipid contents of the rats on the different diets is shown in table 3. Groups X-1, X-2, X-3, and X-7 did not receive plant sterols in their diets. These groups had fat, bile salts, or both added to their diet. The addition of fat to the basal diet at the 5% level had no effect on the liver lipids, whereas the addition of 25% of oleic acid (X-2) increased the liver sterols approximately 27%. When 1% of sodium taurocholate was incorporated into the basal diet (X-3), the liver sterols increased approximately 75% over that of the control group (X-1). Group X-7 which received

TABLE 3

Liver sterols and lipids

GROUP	LIVER WEIGHT	TOTAL STEROL [1]	STEROLS COLORIMETRIC [1]			STEROLS GRAVIMETRIC [2]	NON-STEROL LIPIDS
			Free	Ester [3]	Total	Total	
	gm	*mg/liver*	*mg %*	*mg %*	*mg %*	*mg %*	*%*
X-1	6.4 ± 0.3	13.3 ± 1.2	169	39	208 ± 16	230	3.3
X-2	8.1 ± 0.7	21.5 ± 3.6	180	85	265 ± 28	277	4.8
X-3	6.3 ± 0.7	22.7 ± 1.9	237	122	359 ± 44	394	3.5
X-4	5.5 ± 0.5	13.5 ± 1.4	185	60	245 ± 16	278	3.8
X-5	5.8 ± 0.3	13.7 ± 1.4	203	33	236 ± 16	293	3.3
X-6	8.6 ± 0.3	19.4 ± 1.8	183	43	226 ± 19	237	4.3
X-7	9.5 ± 1.1	38.6 ± 8.3	229	277	406 ± 124	481	4.5
X-8	9.2 ± 1.4	31.1 ± 19.0	202	136	338 ± 167	365	4.8
X-9	5.5 ± 1.5	12.1 ± 1.2	180	40	220 ± 31	242	3.4

[1] Lieberman-Burchard positive digitonin precipitable sterols.

[2] Digitonide weighed.

[3] Expressed as free sterol.

25% of oleic acid and 1% of sodium taurocholate exhibited the greatest rise in liver sterols, approximately 114%.

Groups X-4, X-5, X-6, and X-8 were fed diets comparable to the previous groups, but with 2% of soybean sterols added. The livers of groups X-4, X-5, and X-6 had about the same sterol content. Group X-8 exhibited an increase in liver sterol of approximately 80% when compared to the basal group (X-1). In each of these groups, the liver sterol content was

TABLE 4

Chromatography of serum, liver and fecal sterols

STEROLS	STEROLS Rf	GROUP X-7 Rf	GROUP X-8 Rf
Serum sterols		0.31	0.30
Serum + soy sterols [1]			0.31
			0.0
Liver sterols		0.32	0.33
Liver + soy sterols [1]			0.29
			0.0
Feces		0.30	0.30
			0.0 [2]
Cholesterol	0.33		
7-Dehydrocholesterol	0.43		
Sitosterol	0.0		
Stigmasterol	0.0		
Soy sterols	0.0		

[1] Two and five-tenths micrograms soy sterols added to 47.5 μg serum or liver sterols.

[2] Predominantly plant sterol.

less than the corresonding groups not fed soybean sterols. The increases in liver sterols observed in all of the groups was predominantly in the ester fraction. The other liver lipid fractions increased slightly in the groups receiving oleic acid in their diet.

The values for sterols obtained by the gravimetric procedure were slightly higher in all groups (about 5%) than those observed when the colorimetric method was used. This difference can be attributed to the presence of saturated sterols.

Chromatography. Table 4 gives the observed Rf values for the pure sterols and the serum, liver, and fecal sterols of groups X-7 and X-8. When the serum and liver sterols of group X-8 (1% of bile salts and 2% of soybean sterols) were chromatographed, no plant sterols could be detected. Only one spot with an Rf the same as cholesterol was observed upon chromatographing the liver and serum sterols of both groups. The fecal sterols of this group showed one spot with an Rf value identical with cholesterol, while those of group X-8 gave two spots, one at the origin (plant terol) and the other at Rf 0.30 (cholesterol). The spot at the origin was much larger and more intense than the other, suggesting that the fecal sterols of group X-8 were predominantly plant sterols.

DISCUSSION

The available evidence suggests that there are at least 4 main factors involved in the absorption of sterols; namely, a fatty acid source, bile, pancreatic cholesterol esterase, and the sterol. It has been demonstrated that fat facilitates the absorption of cholesterol and that fatty acid is the active factor (Kim and Ivy, '52; Swell et al., '55). In this connection oleic acid was found to be the most effective fatty acid in promoting cholesterol absorption. The role of dietary fat in the absorption process is two-fold (Swell et al., '55); it provides the fatty acid necessary for cholesterol esterification in the intestine and stimulates the flow of bile. Bile has been shown to be essential for the absorption of cholesterol (Chaikoff et al., '52) and its action would appear to be related to the requirement of bile salt for cholesterol esterase activity (Swell et al., '53). Pancreatic juice has recently been demonstrated to be essential for cholesterol absorption and its requirement is related to the presence of pancreatic cholesterol esterase (Hernandez et al., '55; Swell et al., '54b).

The data obtained in the present study show very clearly that plant sterols may be absorbed in comparatively large amounts and that the same conditions are required as for the absorption of cholesterol. Maximum plant sterol absorption

occurred when 25% of oleic acid and 1% of sodium taurocholate were added to the basal diet. The plant sterols could be virtually all recovered from the feces when incorporated into a fat-free diet. When bile salts were added to a fat-free diet appreciable plant sterol absorption occurred (15.7%). Similar findings have been observed with respect to cholesterol (Swell et al., '55), in that the fatty acid necessary for cholesterol esterification in the intestine can come from endogenous sources. Also, sitosterol, stigmasterol, and cholesterol are esterified *in vitro* by pancreatic cholesterol esterase in the presence of bile salts and fatty acid. Thus, the available evidence suggests that plant sterols are absorbed from the intestine by the same mechanism as cholesterol.

Recent studies (Cook et al., '54; Gould, '54; Hanahan and Wakil, '53) have also demonstrated that dihydrocholesterol and ergosterol are absorbed. These sterols are also esterified *in vitro* which is further evidence of the importance of esterification as a step in sterol absorption.

The liver sterol data indicate that, although large excesses of plant sterols were present in the diet and up to 22.9% of the amount fed was absorbed, there was at most only a small rise in the liver sterols of the group (X-8) absorbing the greatest amount of plant sterols. It should be emphasized that the changes observed in the liver sterols in the animals not receiving plant sterols represent endogenous sterol changes. The increases observed when bile salts, fat, or both were present in the diets of these animals may be ascribed to more efficient reabsorption of the rats' own cholesterol supply. When plant sterols were included in the diet, it is presumed that they inhibited and reduced the absorption of the rats' own cholesterol supply. Therefore, the slight increase in liver sterols observed in group X-8 can be attributed to the effect of the absorbed dietary plant sterols. However, when the liver sterols of this group were chromatographed, no plant sterols were detected.

The serum changes were unexpected since the feeding of plant sterols has been reported to have lowered the blood

cholesterol in animals on cholesterol diets. Also, Gould ('54) reported that labeled plant sterols disappeared rapidly from the blood after their absorption; they did not accumulate in serum or liver. However, in those earlier studies, cholesterol-free diets containing large amounts of plant sterols were not fed nor was the feeding carried out for at least three weeks as in the present study. The greatest rise in serum sterols occurred in the group (X-8) which absorbed the greatest amount of plant sterols. The increase in serum sterols observed in this group cannot be attributed to accumulation of plant sterols in serum, since, as demonstrated by chromatography, less than 5% of the serum sterols were plant sterols. The increases were due to cholesterol or a sterol with the same Rf as cholesterol.

The virtual absence of plant sterols in the serum and liver of rats absorbing large amounts (40 mg/day) of these sterols suggests that: (1) these sterols are rapidly removed from the serum and degraded, probably to cholic acid, by the liver; (2) are transformed during absorption or in the liver to a compound or compounds indistinguishable from cholesterol by the methods used in this study.

SUMMARY

Rats were fed synthetic diets containing 25% of oleic acid, 1% of sodium taurocholate, and 2% of soybean sterols in various combinations. The sterol intake was accurately measured and fecal sterol excretion was determined gravimetrically. On a fat-free diet virtually all of the plant sterols could be quantitatively recovered in the feces. When 1% of sodium taurocholate was added to the fat-free diet, 15.7% of the sterols ingested were absorbed (25 mg/day). Addition of 25% of oleic acid to the basal diet also promoted the absorption of the plant sterols (15.5% of the amount fed, or 24 mg/day). Maximum plant sterol absorption, 22.9% of the total amount fed (40 mg/day), occurred when 25% of oleic acid and 1% of sodium taurocholate were added to the basal diet.

The absorbed plant sterols had very little effect on the liver sterols. At most a slight increase was observed in the group exhibiting maximum plant sterol absorption.

The serum sterols increased in the groups fed plant sterols with oleic acid or oleic acid and bile salts. The addition of oleic acid and bile salts to the diet produced the highest elevation of the serum sterols (104.4 mg %).

No plant sterols could be detected by chromatography (at the 5% level) in the groups fed plant sterols.

The data in the present study suggest that plant sterols are absorbed through the same mechanism as cholesterol and that plant sterols may be conyerted to cholic acid, cholesterol, or a cholesterol intermediate in the intestine or liver.

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PLACENTAL TRANSFER OF CALCIUM^{45} IN THE RAT[1]

J. P. FEASTER, SAM L. HANSARD,[2] J. C. OUTLER AND G. K. DAVIS
Nutrition Laboratory, Florida Agricultural Experiment Station, Gainesville

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Radioactive calcium was introduced into placental transfer studies by Pecher and Pecher ('41). Using mice, these workers noted that frequently the concentration of radioactive calcium in the whole fetus exceeded that in the bones of the maternal animal. Plumlee et al. ('51), studying the placental transfer of Ca^{45} in cattle, found that the ratio of maternal-to-fetal blood Ca^{45} decreased from 20 to 1 at 10 minutes after isotope injection to 1.5 to 1 after 30 hours. These same investigators ('52) observed a decrease in the specific activity (ratio of the concentration of Ca^{45} to the concentration of total calcium) of fetal bones with increasing fetal age. They attributed this to a dilution of the radioactive calcium by the increasing total calcium content of the bones.

The present study was undertaken to investigate the behavior pattern of Ca^{45} in the rat as a function of fetal age and time after isotope administration.

EXPERIMENTAL

Seventy-two nulliparous rats 12 to 15 months of age maintained on a diet of commercial rat pellets containing 1.38%

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[2] Present address: U.T. — A.E.C. Agricultural Research Program, Oak Ridge, Tennessee.

of calcium and 0.96% of phosphorus were bred and at definite stages of gestation were given orally single tracer doses of Ca^{45} chloride [3] (3 μg containing about 0.12 mg calcium) and placed in individual metabolism units (Hansard and Comar, '53) for routine balance studies. The pregnant rats were sacrificed after intervals of from one-half hour to 20 days following isotope administration and the fetuses and carcasses of the dams were analyzed for radiocalcium and total calcium content. Excretions were assayed for Ca^{45} activity only. Tissue samples were prepared for analysis by ashing in weighed crucibles, then dissolving the ash in dilute hydrochloric acid; aliquots of this solution were taken for determinations of Ca^{45} and total calcium content. Calcium^{45} activity was determined by precipitating the calcium as the oxalate, filtering the oxalate on tared discs and placing these discs under a mica end window Geiger Mueller tube for counting. Total calcium determinations were carried out by a modification (McCall, '54) of the method of Brunisholz and co-workers ('53). Calcium was precipitated quantitatively as the oxalate from an aliquot of the tissue sample solution, then the precipitate was redissolved in hydrochloric acid and the pH of the solution adjusted to 9. After addition of a measured volume of a standard solution of magnesium sulfate the calcium-magnesium mixture was titrated with ethylene diamine tetra acetic acid,[4] using eriochrome black T as indicator.

RESULTS AND DISCUSSION

The weight and ash content of fetuses from 14 days of gestation to 22 days, or term, are shown in table 1.

An increase in fetal weight of about 55 times was noted from 14 to 22 days. Ash, or mineral concentration, increased during this period from 1.1% at 14 days to 1.7% at 22 days, a gain which appears rather slight. However, this repre-

[3] The Ca^{45} chloride was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee, after allocation by the Isotope Division of the Atomic Energy Commission.

[4] Versenate.

sents an increase of from 0.001 gm of ash per fetus at 14 days to 0.093 gm per fetus at 22 days, or an increase of 93 times in weight of the ash in the fetus.

Transfer of orally administered calcium[45] as a function of fetal age. Table 2 shows the relationship between fetal age and the transfer of calcium to the fetus. All data presented

TABLE 1

Weight and ash content of fetuses as a function of age

Fetal age, days	14	15	16	17	18	19	20	21	22
No fetuses	45	33	22	18	15	14	30	37	65
Av. fresh wt., gm	0.10	0.14	0.25	0.62	1.52	2.74	3.83	4.93	5.57
Ash, %	1.1	1.1	1.2	1.2	1.4	1.4	1.5	1.6	1.7

TABLE 2

Calcium transfer as a function of fetal age [1]

FETAL AGE	TOTAL Ca	CALCIUM[45]		SPECIFIC ACTIVITY [3]
days	*mg/gm fresh wt.*	*% Administered dose/litter*	*% Retained dose/litter* [2]	
14	0.18	0.7	2.1	16.8
15	0.30	1.2	3.4	10.2
16	0.35	1.9	5.4	8.8
17	0.74	4.2	11.3	2.1
18	1.01	7.5	18.5	1.3
19	1.88	11.9	26.6	0.9
20	2.12	18.9	36.5	0.8
21	1.77	18.9	36.5	0.6
22	2.21	22.4	40.4	0.6

[1] Maternal animals sacrificed 96 hours after isotope administration.

[2] $\frac{\%\text{ total dose per litter}}{\%\text{ total dose retained, dam and fetuses}} \times 100.$

[3] $\frac{\%\text{ retained dose per gram fresh weight}}{\text{mg Ca per gram fresh weight}}.$

in this table were obtained on fetuses from dams dosed orally with Ca^{45} and sacrificed 96 hours after isotope administration.

Total calcium values indicate the progressive mineralization taking place during fetal development. Calcium concentrations increased markedly at 18 days with a slight decrease

apparent at 21 days, and peak concentration was reached at term. The increase in total ash per fetus by a factor of 93 times during the period from 14 to 22 days (table 1) was exceeded several times over by the gain in total calcium per fetus, the latter value increasing by 676 times during the same period.

Calcium^{45} values are expressed as percentage of the total administered dose per litter, the latter value computed by dividing the percentage of the total administered dose found in the litter by the percentage of the total dose retained (percentage found in fetuses and carcass of the dam less gastrointestinal tract and its contents) and multiplying the quotient by 100. The percentage retained dose per litter value thus represents the part of the potentially available Ca^{45} transferred to the fetuses. A steady increase in percentage total dose per litter with increasing age was noted, with approximately 32 times as much Ca^{45} transferred to fetuses from dams sacrificed at 22 days gestation as was transferred to those fetuses from dams sacrificed at the 14-day stage. A similar increase noted in the values for percentage *retained* dose per litter with increasing fetal age is due entirely to the transfer of a larger fraction of the available Ca^{45} during a 96-hour interval in the latter stages of gestation than during the same interval in the earlier stages, the amount of Ca^{45} available being approximately the same in all the dams involved. Despite this increase in percentage retained dose, fetal specific activity (percentage retained Ca^{45} dose per gram of fresh weight divided by milligrams of calcium per gram of fresh weight) decreased as fetal development progressed. This is probably due to the fact that while Ca^{45} (stable, dietary calcium) was ingested continuously during the 96-hour period between isotope administration and sacrifice, Ca^{45} was ingested only at the beginning of the period. This continuous availability of Ca^{40} had the effect of reducing the value of the Ca^{45}/Ca^{40} ratio as the rate of transfer of all calcium increased with the age of the fetus. Fetal specific activities far exceeded maternal spe-

cific activities, ranging from 672 times as high at 14 days to 23 times as high at 22 days.

Total calcium concentration in the carcass of the dam was found to be constant throughout the gestation period. This observation is in keeping with the findings of Plumlee and collaborators ('52) who noted no effect of the stage of gestation on the behavior of calcium in heifers carrying three-, 6-, and 8-month fetuses. Olsen ('39) calculated from chemical analysis that during pregnancy, mice lost approximately 5 mg of calcium, or 2% of the total reserve, to the fetus.

TABLE 3

Calcium^{45} transfer as a function of time after isotope administration [1]

HOURS AFTER ADMINISTRATION	% OF ADMINISTERED Ca^{45}		TOTAL ADMINISTERED Ca^{45} RETAINED	DISTRIBUTION OF RETAINED DOSE	
	Dam [2]	Litter		Dam	Litter
			%	%	%
0.5	49	13.7	62.7	78.3	21.7
1	61	24.4	85.4	71.3	28.7
5	51	24.0	74.9	67.8	32.2
24	45	25.2	70.2	64.3	35.7
96	42	23.5	65.5	64.3	35.7
120	30	17.4	47.4	62.9	37.1
480	28	16.1	44.1	63.6	36.4

[1] Maternal animals in last fifth of gestation period at time of sacrifice.

[2] Carcass less gastro-intestinal tract and contents.

Transfer of orally administered calcium^{45} as a function of time after dosing. The data in table 3 illustrate the transfer of Ca^{45} to the fetus as a function of time after oral administration. The dams from which these fetuses were taken had received oral doses of Ca^{45} at a given time during the gestation period ranging from one-half to 480 hours prior to sacrifice. All of the females were sacrificed between the 19th and the 22nd day of the gestation period.

The rapidity with which transfer of orally administered Ca^{45} takes place is indicated by the fact that in only one-half hour after dosing, 13.7% of the total amount of Ca^{45} administered, or 21.7% of the Ca^{45} available for transfer (retained dose) had crossed the placenta to the fetuses.

The percentage of the total Ca^{45} dose retained by the dam increased up to one hour after isotope administration. Beginning with the dams sacrificed at 5 hours after isotope administration a steady decrease in the amount retained was noted as the rate of reexcretion of Ca^{45} into the intestine and transfer to the fetuses exceeded the rate of absorption. The percentage of the total Ca^{45} dose present in each litter of fetuses also increased up to one hour after administration of the isotope; thereafter it remained constant for litters from maternal rats which had received Ca^{45} up to 96 hours prior to sacrifice. In litters from animals which had received the dose 120 and 480 hours prior to sacrifice, the percentage of the total dose present was lower than in litters from animals which had received the dose one hour prior to sacrifice, when all were sacrificed between the 19th and 22nd day of pregnancy. This is probably not the result of the return of Ca^{45} across the placenta to the maternal animal, but rather of the lower level of Ca^{45} available for transfer in the tissues of these dams which received Ca^{45} earlier in the gestation period, by the time that stage of fetal development was reached at which comparatively rapid transfer of calcium takes place. The 120- and 480-hour percentage retained dose per litter values, in the computation of which the percentage of the dose present in the dam is taken into account, did not show this decrease. This is a further indication that the reduction in percentage dose per litter is a matter of reduced maternal Ca^{45} level.

As noted in table 2, when rats were dosed orally with Ca^{45} at 10 to 14 days gestation and sacrificed 96 hours later (at 14 to 18 days gestation), from 0.7 to 7.5% of the dose was transferred to the fetuses. The values in table 3 show that when rats were dosed on the first or second day and sacrificed 20 days (480 hours) later, about 16% of the administered Ca^{45} was transferred to the fetuses. Thus in the case of these rats dosed very early in pregnancy, the greater part of the amount of Ca^{45} transferred would appear to have passed to the fetuses after the 18th day. These results indicate that a

portion of the calcium which crosses the placenta to the developing young must be calcium which has previously been stored in the tissues, chiefly the bones, of the maternal animal. Further evidence in support of this concept is found in the results of administering Ca^{45} to female rats prior to conception. Fetuses from rats dosed two days before conception and sacrificed 18 days later were found to have slightly higher specific activities than the maternal animals, the fetal-to-maternal specific activity ratios averaging 1.24. When rats were dosed 15 days before conception and sacrificed 22 days after conception, this ratio was found to be 1.14. (Fetal-to-maternal specific activity ratios in the time study represented by table 3 ranged from 13 for the rats receiving Ca^{45} one-half hour prior to sacrifice to 28 for those dosed 480 hours prior to sacrifice.)

SUMMARY

Calcium^{45} administered orally to pregnant rats was found to cross the placenta and deposit in the fetus at all stages of gestation studied, from the 14th to the 22nd day. The amount of labeled calcium transferred from dam to fetus in a given length of time after administration increased with increasing fetal age. A study of Ca^{45} transfer as a function of time after isotope administration revealed that in one-half hour an appreciable amount of the orally administered isotope had been absorbed and transferred to the fetus. The findings indicate that a part of the calcium transferred to the fetus must have been derived from calcium previously stored in the maternal skeleton.

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INFLUENCE OF DIETARY FIBER ON METABOLIC AND ENDOGENOUS NITROGEN EXCRETION

JAMES H. MEYER
Animal Husbandry Department, University of California, Davis

TWO FIGURES

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The influence of dietary fiber on metabolic fecal nitrogen has an important bearing on calculations of true digestibility and biological value of proteins in fibrous feeds. Mendel and Fine ('12) noted an increase in metabolic fecal nitrogen when the indigestible material in the diet was high and suggested that the nitrogen-free diets be compounded to contain the same amount of indigestible material as the test diet when true digestibility was to be determined. Evidence was presented by Mitchell ('24) that consumption of undetermined amounts of filter paper increased metabolic nitrogen in the feces. Schneider ('34), in his extensive compilation of data on metabolic fecal nitrogen, noted that indigestible material in the diet increased metabolic fecal nitrogen but did not change the slope of the curve when metabolic fecal nitrogen was plotted against food intake. He also warned that it was important to have the same amount of indigestible material in the nitrogen-free or the nitrogen-low diet as in the test diet. Investigations have not been made to study specifically the relationship of dietary fiber and metabolic fecal nitrogen. The experiment reported herein was designed to study this relationship.

EXPERIMENTAL

The experiment consisted of a nitrogen balance study on rats with the following additions to the ration: 0, 5, 15, and

30% cellulose.[1] Cellulose was added at the expense of the basal ration, (table 1) so that the ratios of sucrose and fat to protein, vitamins and minerals remained constant. The basal raton contained 0.52% of nitrogen. Twelve replications were used per treatment. All animals within a replication were fed daily equal amounts of the basal ration plus their assigned cellulose increment. The food consumption of the rat fed the 30% cellulose ration within a replication was measured daily. Then an amount of each of the other rations

TABLE 1

Nitrogen-low basal ration

INGREDIENT	NITROGEN-LOW DIET
	gm
Sucrose	86.8
Defatted whole egg protein [1]	4.0
Cottonseed oil [2]	5.0
Salts IV [3]	4.0
Vitamin mix [4,5]	0.1
Choline	0.1
	100.0

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

[2] Wesson oil.

[3] Phillips and Hart, J. Biol. Chem., *109:* 657, 1935.

[4] The vitamin mix supplied the following vitamins in milligrams per 100 gm of the ration: thiamine 0.3, riboflavin 0.3, pyridoxine 0.2, niacin 0.4, calcium pantothenate 4.0, folic acid 0.5, biotin 0.02, and vitamin B_{12} 0.002.

[5] Ample amounts of vitamins A, D, E, and K were supplied weekly by oral administration.

(0, 5, or 15% cellulose) which would contain an equivalent amount of the basal ration, was weighed and fed to the remaining rats in the replication.

Male, weanling, Sprague-Dawley rats were used. They were housed in screened-bottom cages for the first 7 days. The metabolism cages designed from the description of Harned et al. ('49) were then used for the 7-day collection and metabolism study. These cages prevented the contamination of feces with urine. Nitrogen was determined by the Kjeldahl

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

method. Analysis of variance as described by Snedecor ('46) was used as the test of significance. The method of least significance was used to determine differences between treatments and the controls.

RESULTS AND DISCUSSION

Table 2 represents the results of this experiment. All animals in the 4 treatments consumed practically equal amounts

TABLE 2

Influence of cellulose intake on metabolic and endogenous nitrogen excretion

ITEMS COMPARED	0% CELLULOSE	5% CELLULOSE	15% CELLULOSE	30% CELLULOSE
Rats per group	12	12	12	12
Average wt., gm	64	63	63	65
Daily food consumption:				
Basal ration, gm	3.41	3.43	3.39	3.41
Cellulose, gm		0.18	0.60	1.46
Endogenous nitrogen, mg per gm wt.$^{0.75}$	1.46	1.31	1.50	1.38
Metabolic nitrogen				
Total excreted, mg per day	4.56	5.19	6.98 [1]	8.81 [1]
Mg per gm of total food intake	1.38	1.44	1.75 [2]	1.81 [2]
Mg per gm of feces	34.5	17.9 [1]	10.0 [1]	5.9 [1]
Indigestible dry matter, %	3.82	8.24 [1]	16.69 [1]	31.26 [1]

[1] Indicates a highly significant difference from 0% cellulose.
[2] Indicates a significant difference from 0% cellulose.

of basal ration and hence had the same nitrogen intake. Therefore, any differences between treatments are due to cellulose consumption.

Endogenous urinary nitrogen excretion was not influenced by cellulose intake.

Excretion of metabolic nitrogen in the feces was influenced by cellulose intake. Each added increment of cellulose in-

creased total fecal nitrogen excretion. The additional loss of metabolic nitrogen in the feces from consumption of large quantities of indigestible fiber might be expected, therefore, to increase protein requirement of animals fed rations high in indigestible dry matter. This has been emphasized for ruminants by Blaxter and Mitchell ('48).

When metabolic fecal nitrogen excretion was expressed on the basis of food consumption, significant differences between groups were noted. This offers direct evidence for the state-

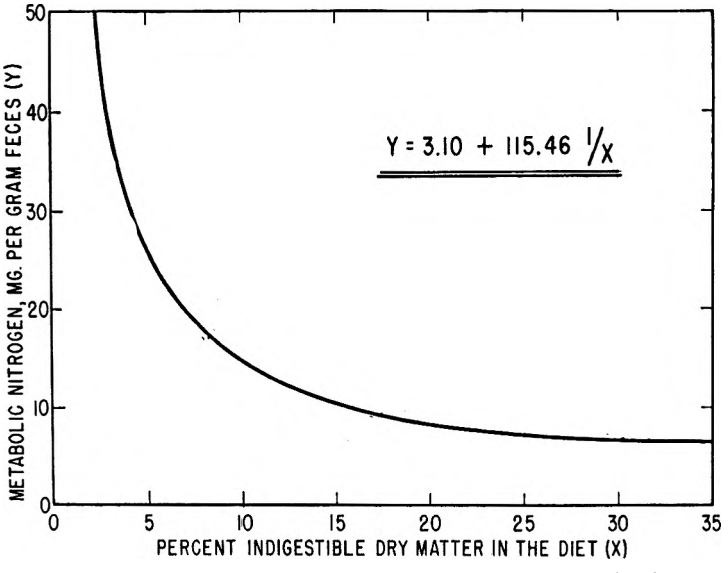

Fig. 1 The regression of the ratio of metabolic nitrogen to fecal dry matter on indigestible dry matter in the diet. Each point is an observation of one rat.

ment of Mendel and Fine ('12) and Schneider ('34) that it is important in biological value and true digestibility determinations to have the same fiber content in the test and nitrogen-low rations.

An inspection of the data showed that the fecal nitrogen concentration and either the cellulose intake or indigestible dry matter were inversely related. Less variation was present when metabolic fecal nitrogen concentration was related to the indigestible dry matter then when related to the dietary

cellulose levels. This was true because individual rats showed differences in the digestibility of the rations. Figure 1 shows graphically the fecal nitrogen concentration relationship to indigestible dry matter. These data are best described by the regression equation

$$Y = 3.10 + 115.45\ 1/X$$

in which Y is the metabolic nitrogen in milligrams per gram of fecal dry matter and X is the percentage of indigestible

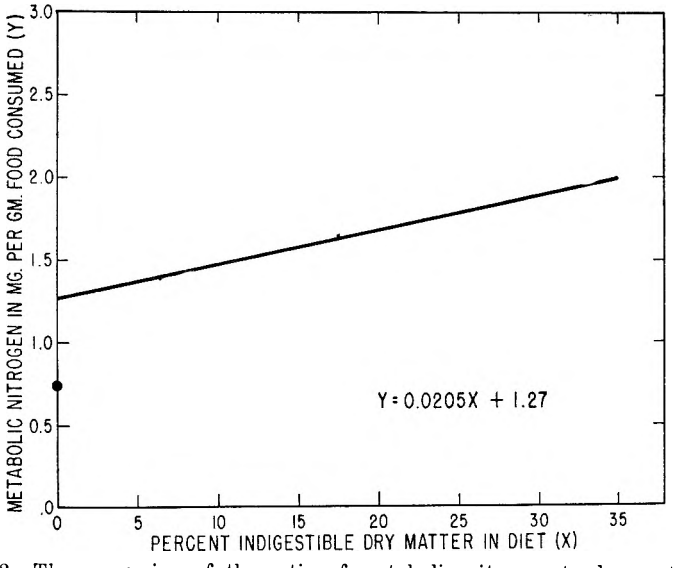

Fig. 2 The regression of the ratio of metabolic nitrogen to dry matter consumed on indigestibility dry matter in the diet. Each point is an observation of one rat.

dry matter in the diet. Both the regression ($b = 115.46$) and the correlation ($r = 0.92$) of milligrams of nitrogen excreted per unit of fecal dry matter on the reciprocal of the percentage of indigestible dry matter were statistically highly significant.

The most commonly used reference base for fecal metabolic nitrogen is food consumption. The relationship of this ratio to indigestible dry mater is shown in figure 2. The regression was a straight line relationship and was highly significant.

The correlation was 0.48. This indicates that food consumption as a reference base for metabolic fecal nitrogen can be used as noted by Mitchell ('24) and Schneider ('34).

However, an inspection of figures 1 and 2 indicates that in this experiment fecal dry matter is more satisfactory than food consumption as a reference base for metabolic nitrogen. As confirmation it was found that the correlation of metabolic fecal nitrogen with food consumption was 0.60 but was 0.78 with fecal dry matter excretion. A similar observation was made earlier for man (Heupke, '33) and cattle (Mukherjee and Kehar, '49).

SUMMARY

The influence of dietary fiber on endogenous urinary and metabolic fecal nitrogen excretion was studied. Nitrogen balance studies were used which involved 48 growing rats fed 0, 5, 15 or 30% cellulose and pair-fed to insure equal intakes of a basal ration containing 4% of whole egg protein plus the respective cellulose levels. There was no influence of fiber intake on endogenous urinary nitrogen excretion. Each added increment of cellulose increased total fecal nitrogen excretion. This relationship to indigestible dry matter in the diet was examined and was best described by a curvilinear regression of metabolic nitrogen per unit of fecal dry matter on indigestible dietary dry matter.

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PROTEIN IN THE NUTRITION OF THE GROWING DOG [1]

C. F. GESSERT [2] AND P. H. PHILLIPS
Department of Biochemistry, University of Wisconsin, Madison

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Our present knowledge of the nutrient requirements for dogs was summarized and discussed in a report of the Committee on Animal Nutrition of the National Research Council ('53). The minimum amounts of protein or amino acids which are required by young, growing dogs have not been established. Heiman ('47) reported that a diet containing 15% of protein (supplied by soybean, meat, and fish meals and flaked cereals) did not permit optimum growth of cocker spaniel pups, whereas a similar diet containing the same ingredients in different proportions, so that the protein content was 20%, did support good growth. In view of the large amount of protein consumed by dogs, it is desirable to know their requirements more accurately.

EXPERIMENTAL

Any consideration of the quantitative requirement for protein should take into account both the qualitative and the quantitative amino acid composition of the protein. In this study, the basal diet was designed to contain 10 to 11% of well balanced protein, i.e., to have no large excess or outstanding deficiency of any essential amino acid or acids. The compositions of the basal diet (no. 1) and the other experi-

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[2] Present address: Dept. of Otolaryngology, School of Medicine, Washington University, St. Louis, Mo.

mental diets are given in table 1. All diets were intended to provide the same adequate amounts of the non-protein nutrients; only the quantities of casein and sucrose were varied. Kjeldahl analyses for protein nitrogen indicated that diet 1 contained 10.6% of crude protein (N × 6.25), and diet 5 contained 19.4%. Correspondingly, diets 2, 3, and 4 contained 12.8 15.0, and 17.2% protein, respectively.

TABLE 1

Percentage composition of diets

INGREDIENT	DIET 1	DIET 2 [1]	DIET 3 [1]	DIET 4 [1]	DIET 5 [1]
	%	%	%	%	%
Skim milk, dried	10.0				
Alfalfa leaf meal	5.0				
Primary brewers' yeast, dried	3.0				
Soybean meal, expeller processed	5.0				
Corn, yellow, no. 2 Dent, ground	25.0				
Sucrose	44.0	41.5	39.0	36.5	34.0
Casein, ethanol extracted [2]	0.0	2.5	5.0	7.5	10.0
Lard, open kettle, leaf	6.0				
NaCl (iodized)	1.0				
$CaHPO_4 \cdot 2H_2O$	1.0				
Vitamin supplements [3]	...				

[1] Diets 2, 3, 4 and 5 have the same composition as diet 1, except for the amounts of sucrose and casein.

[2] Each 100 gm of casein contributed 88 gm of "protein," i.e., N content × 6.25 = 88%. With each 100 gm of casein, 1.8 gm of $CaCO_3$ were added.

[3] The vitamin supplement consisted of the following:
Vitamin A: 1000 I.U./kg diet (Nopco stabilized).
Vitamin D: 900 I.U./kg diet (Irradiated yeast).
Vitamin B_{12}: 10 μg/kg diet.

Eight pups, one from each of 8 litters were assigned to each of 5 experimental groups, with each group designated by the number of the diet which it received. The distribution of pups among the 5 groups was as equal as possible with regard to sex and average starting weights. The pups were housed indoors, one to three per cage, in cages which had raised, expanded metal floors (1″ × 2″ mesh). The animals were given their diets and tap water ad libitum, and a record was kept of the amount of diet consumed by each group. Pups which were

brought into the kennel were dipped into a 1% D.D.T. emulsion bath, then washed with soap, and rinsed. All pups were dewormed prior to the experimental period and were periodically given subcutaneous injections of homologous anti-canine distemper serum and anti-infectious canine hepatitis serum. The two beagle and six mongrel (predominantly collie) pups in each group were started on experiment at 6 to 7 weeks of age, and were kept on their respective diets for 14 weeks or more.

TABLE 2

Data on growth and utilization of the diets (12 weeks)

CATEGORY	GROUP				
	1	2	3	4	5
Per cent dietary protein:	10.6	12.8	15.0	17.2	19.4
Average weights, kg					
Initial [1]	2.14	2.12	2.12	2.12	2.11
Final	5.12	7.36	8.05	8.94	8.75
Gain in 12 wks., av.	2.98	5.24	5.93	6.82	6.64
Comparative gains [2]	43	77	87	100	98
Diet efficiency ratio [3]	0.17	0.24	0.25	0.26	0.27

[1] Initial weight range 1.3 to 3.1 kg.

[2] The percentage gain of one group is arbitrarily set at 100, and the gains of the other groups are compared on this basis.

[3] Utilization of the whole diet expressed as kilograms gain in body weight per kilogram of diet consumed by all pups.

RESULTS

The data on growth for 12 weeks are presented in table 2. Most of the pups in group 1 made slow but steady gains, and the few which did not gain were able to maintain their initial weights and good health. However, the pups in group 2, which received 12.8% protein, made significantly greater gains than those in group 1. Each added portion of dietary protein caused an increase in the average rate of growth until 17.2% protein was reached; an additional amount beyond that caused no further increase. The utilization of diet 2 was much better than that of diet 1, as shown by the marked difference in their diet efficiency ratios, presented in table 2. Further increments

of dietary casein, however, caused only minor additional increases in the diet efficiency ratio.

These data were obtained with dogs whose adult weights were 20 to 25 lb. and 35 to 40 lb. for the beagles and the collies respectively.

One long-haired, male pup on diet 1 increased in weight from 1.95 to 2.45 kg in 16 weeks, whereas his female littermate on diet 5 increased from 2.20 to 10.05 kg during the same period. After 16 weeks, the male pup was transferred to diet 4, and 14 weeks later, he weighed nearly 10 kg. Hence, it appears that a young dog which has been retarded by receiving insufficient dietary protein during the normal growth period is still able to grow normally when his protein requirement is subsequently met. Osborne and Mendel ('16) demonstrated a similar response in rats.

DISCUSSION

The requirements of growing young dogs for individual amino acids have not been established. Madden et al. ('44) reported the amounts of the 10 amino acids (as the DL isomers in 6 cases) which were required for the production of normal plasma protein in an adult dog. These acids were the same as those required by the rat for growth, but the amounts in which they were needed by the adult dog for plasma protein production give no indication of the amounts required for growth. Mitchell ('50) has pointed out that the proportions of the amino acids required for adult maintenance are quite different from the proportions in which they are required for growth by the young animal. During growth, the synthesis of muscle and other soft tissues is dominant in determining amino acid requirements; in the adult animal, however, keratin synthesis (for hair and nails), with a high need for methionine and cystine, and a low need for lysine, histidine, and phenylalanine, dominates the total amino acid requirements.

Estimations were made of the amounts of individual essential amino acids in each of the diets. The results are presented in table 3, together with the requirements of the rat, for com-

parison. Since the pup's requirements seem to have been met satisfactorily by diet 4, it can be assumed that none of the requirements for individual acids exceed the amounts of the acids that were present in this diet, but the amounts undoubtedly exceeded the minimum requirements in several instances. It should be pointed out that the amounts of the amino acids in table 3 are estimates based on literature values of ingredient

TABLE 3

Estimated amino acids in various diets[1] *(per cent)*

CONSTITUENT	DIET 1	DIET 2	DIET 3	DIET 4	DIET 5	RAT REQUIREMENT[2]
	%	%	%	%	%	%
Protein	10.6	12.8	15.0	17.2	19.4	
Lysine	0.62	0.81	0.99	1.18	1.37	1.0
Phenylalanine + tyrosine	1.02	1.20	1.58	1.86	2.14	0.9
Phenylalanine only	(0.50)	(0.64)	(0.77)	(0.91)	(1.05)	
Leucine	1.02	1.24	1.46	1.68	1.90	0.8
Valine	0.66	0.83	1.00	1.17	1.34	0.7
Methionine + cystine	0.34	0.43	0.51	0.60	0.69	0.6
Methionine only	(0.19)	(0.27)	(0.34)	(0.42)	(0.50)	
Isoleucine	0.53	0.70	0.86	1.03	1.19	0.5
Threonine	0.41	0.51	0.61	0.71	0.81	0.5
Histidine	0.28	0.35	0.42	0.49	0.57	0.4
Tryptophan	0.13	0.16—	0.18+	0.21	0.24	0.2
Arginine	0.47	0.56	0.66	0.75	0.84	0.2

[1] The estimations are based on the amino acid contents of the feed ingredients, as compiled by Sievert and Fairbanks ('55), and on the amino acid composition of casein, as given by Block and Bolling ('51).

[2] The rat requirements for growth are as suggested and modified by Rose and associates (Rose et al., '49; Wretlind and Rose, '50).

analyses, and that the acids are probably not all completely available for anabolism by the dog. According to the table, diet 4 completely satisfied or exceeded all of the rat requirements, whereas in diet 3, only the amounts of methionine and of tryptophan did not meet these requirements.

When the percentage of each acid in diet 1 was calculated with respect to the amount of that acid in diet 4, the average of the 10 acids was 57%, with a range from 51% for isoleucine to 63% for arginine. The averages for diets 2 and 3 were 72

and 86% respectively, whereas the amounts of total protein in diets 1, 2, and 3 were 62, 74, and 87% of the amount in diet 4. The comparative gains (table 2) on the 4 diets were: 43, 77, 87, and 100%. The marked improvement in growth on diet 2 over that on diet 1 was greater than the increase in total protein or in essential amino acids in diet 2 as compared to diet 1, thus indicating that a nutritive critical point existed in the difference between the two diets. This indication is further borne out by the fact that there was a large improvement in diet utilization when the first increment of casein was added to the basal diet, whereas further improvement in diet utilization with additional protein was only slight. Besides the likelihood that critical concentrations of certain amino acids were attained in diet 2, it is also probable that the addition of casein to the diet increased the utilization of the protein which was already present in the basal diet.

SUMMARY

The protein requirement for growth of young dogs was investigated by adding increments of casein to a basal diet which contained 10.6% of protein (N $\times$ 6.25), and was adequate in other nutrients. The other 4 diets contained 12.8, 15.0, 17.2 and 19.4% of crude protein respectively.

The basal diet sustained health and a retarded rate of growth in the young dogs. There was a correlation between the comparative gains in body weight and the comparative protein contents of the diets from 12.8 to 17.2% of protein, beyond which point there was no further increase in growth rate caused by additional protein. Since the diet containing 17.2% of protein was adequate for the growth of the pups, their requirements for the 10 essential amino acids apparently do not exceed the amounts that it contained.

The utilization of the basal diet was substantially improved by the addition of 2.5% of casein (2.2% "protein"), but more casein caused very little further improvement in this respect.

A 5½-month-old pup which had been very retarded in growth for 4 months by a protein deficiency was still able to grow at a normal rate when his dietary protein was raised to 17.2%.

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ADVERSE EFFECTS OF SOME AMINO ACID SUPPLEMENTS IN LOW-PROTEIN DIETS FOR GROWING DOGS [1]

C. F. GESSERT [2] AND P. H. PHILLIPS
Department of Biochemistry, University of Wisconsin, Madison

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Several attempts to improve the growth-supporting properties of low-protein diets for dogs have shown that the addition of certain amino acids caused a decrease, rather than an increase, in the rate of growth. These decreases were obtained with amounts of lysine and methionine that would not ordinarily be considered excessive. The two experiments reported here are from a study whose purpose was to gain information that would be useful in establishing the protein required for the satisfactory growth of the pups of medium-sized breeds (adult weight 25 to 45 lbs.). These experiments were selected because they demonstrate the adverse effects of amino acid supplementations.

EXPERIMENTAL AND RESULTS

Each group was composed of 8 or 9 collie-shepherd pups. In each experiment, littermates were distributed equally among the groups, and the distribution of the pups was as equal as possible with regard to sex and average starting weights. Both basal diets were designed to contain adequate amounts of the non-protein nutrients. The pups were pre-

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[2] Present address: Dept. of Otolaryngology, School of Medicine, Washington University, St. Louis, Mo.

pared for experiment, housed and fed as reported previously (Gessert and Phillips, 56). In experiment A, the pups were periodically given subcutaneous injections of homologous anti-canine distemper serum and anti-infectious canine hepatitis serum. The pups in experiment B were vaccinated subcutaneously with canine distemper vaccine and infectious canine hepatitis vaccine in bronchisepticus-streptococcus-typhimurium bacterin, for more complete protection.

Experiment A. An attempt was first made to improve a low-protein basal diet, no. 11, by the addition of several amino acids which were thought to be present in insufficient amounts. The compositions of diets 11 through 15 are given in table 1. Kjeldahl analyses indicated that diet 11 contained 10.6% of crude protein ($N \times 6.25$), and diet 15 contained 19.0%. The starting ages of the pups varied from 7 to 10 weeks.

The data on the average growth rates are presented in table 2. In 10 weeks, the pups in group 11 made an average gain which was 60% of that made by their littermates fed diet 15. The supplement of 0.4% of L-lysine added to the basal diet caused a definite decrease in the rate of growth. When 0.3% of DL-methionine was added with the 0.4% of lysine, the depression caused by lysine was corrected, but the growth rate was not greatly improved over that of the dogs fed the basal diet alone. The addition of 0.13% of L-histidine along with the lysine and methionine caused only a slight further improvement in growth. The diet efficiency ratio of the basal diet was improved about 32% by the addition of 10% of casein The addition of lysine alone to the basal diet caused a decrease in utilization, whereas the combinations of lysine and methionine, or lysine, methionine and histidine, tended to increase it slightly.

Experiment B. The basal diet used in this experiment had many ingredients in common with those of diet 11. The main difference was that diet 21 contained corn flakes in place of all of the whole corn and soybean oil meal and much of the sucrose in diet 11. Lard was used as the fat instead of Crisco. The dietary compositions are presented in table 1. Kjeldahl

analyses showed the crude protein content of diet 21 to be 11.0 to 11.2%. The pups were started on experiment at 6 to 7 weeks of age, and they remained healthy throughout the experiment.

TABLE 1

Percentage composition of diets

CONSTITUENT	DIET 11	DIET 21
	%	%
Skim milk, dried	10.0 (3.4)[1]	9.0 (3.1)[1]
Alfalfa leaf meal	5.0 (1.0)	5.0 (1.0)
Primary brewers' yeast, dried	3.0 (1.4)	3.0 (1.4)
Soybean meal, expeller processed	5.0 (2.2)	
Corn, yellow, no. 2 Dent, ground	25.0 (2.5)	
Corn flakes (protein min. 8%)		65.0 (5.5)
Sucrose[2]	44.0	9.0
Hydrogenated vegetable oil[3]	6.0	
Lard, open kettle, leaf		5.0
NaCl (iodized)	1.0	1.0
$CaHPO_4 \cdot 2H_2O$	1.0	1.25
KCl		0.25
Dextrin (as a carrier)		1.5
Vitamin supplement[4]	..	..

Diet 12: Same as diet 11 plus 0.4% L-lysine (as 0.5% L-lysine·HCl)
Diet 13: Same as diet 12 plus 0.3% DL-methionine
Diet 14: Same as diet 13 plus 0.13% L-histidine (as 0.2% L-histidine·HCl·H_2O)
Diet 15: Same as diet 11 plus 10% casein
Diet 22: Same as diet 21 plus 0.15% DL-methionine
Diet 23: Same as diet 22 plus 0.13% L-lysine (as 0.16% L-lysine·HCl)
Diet 24: Same as diet 22 plus 0.10% DL-tryptophan
Diet 25: Same as diet 23 plus 0.10% DL-tryptophan

[1] The figures in parentheses are estimates of the amounts of protein contributed to the diet.

[2] Supplements of vitamins, amino acids and casein were added at the expense of sucrose.

[3] Crisco.

[4] The vitamin supplement for diet 11 consisted of the following:
Vitamin A: 1000 I.U./kg diet (Nopco stabilized).
Vitamin D: 900 I.U./kg diet (Irradiated yeast).

The vitamin supplement for diet 21 consisted of the following:
Vitamin A: 5000 I.U./kg diet.
Vitamin D: 900 I.U./kg diet.
Vitamin B_{12}: 10 μg/kg diet.

TABLE 2

Data on growth and utilization of the diets

CATEGORY	EXPERIMENT A (10 WKS.) Group 11	12	13	14	15	EXPERIMENT B (12 WKS.) Group 21	22	23	24	25
Average weights, kg										
Initial [1]	2.67	2.60	2.61	2.55	2.55	2.52	2.50	2.53	2.52	2.51
Final	6.25	5.14	6.50	6.71	8.52	5.68	4.49	7.51	5.22	7.61
Gain in 10 wks., av.	3.58	2.54	3.89	4.16	5.97	3.16	1.99	4.97	2.70	5.10
Comparative gains [2]	100	72	112	122	175	100	64	158	86	162
Diet efficiency ratio [3]	0.22	0.19	0.23	0.23	0.29	0.20	0.16	0.25	0.18	0.24
Relative efficiency of diet utilization [4]	100	85	105	105	132	100	80	125	90	120

[1] Initial weight range: 1.7 to 3.8 kg in experiment A and 1.3 to 4.1 in experiment B. Average of 8 dogs in each experiment.

[2] The percentage gain of one group is arbitrarily set at 100, and the gains of the other groups are calculated on this basis.

[3] Expressed as average kilograms gain in body weight per kilogram diet consumed.

[4] The diet utilization of one group is arbitrarily set at 100, and the utilizations by the other groups are compared.

The data on average weight gains are summarized in table 2. The rate of growth was definitely decreased when 0.15% of DL-methionine was included as the only amino acid supplement to the basal diet. When 0.13% of L-lysine and 0.15% of methionine was added simultaneously to the basal diet, the growth obtained (group 23) was 58% better than that obtained in group 21. Diet 24, the basal diet plus 0.10% of DL-tryptophan and 0.15% of methionine, supported better growth than diet 22, but 14% less than the basal diet alone. The combination of methionine, lysine and tryptophan as supplements (group 25) yielded very little further improvement in growth over that obtained with the supplements of methionine and lysine without tryptophan.

DISCUSSION

The essential amino acid contents of the basal diets were estimated by using literature values of amino acid compositions, despite the limitations of such figures. Since the requirements for individual amino acids for growth by the dog are not established, the requirements for growing rats were used as a guide to see how well the basal diets in these experiments satisfied growth requirements (see table 3). Both basal diets appeared to be low in their content of lysine, methionine plus cystine, and tryptophan, and perhaps also of histidine and threonine. When enough L-lysine was added to diet 11 to presumably just meet the rat requirement, the rate of growth, which was already sub-optimal, was further depressed. When enough DL-methionine was added to diet 21 to more nearly meet the rat requirement, the rate of growth of pups was also decreased by this addition of a single amino acid. In both cases the combination of lysine plus methionine was able to overcome the repression in growth caused by either acid alone. However, when they were added to diet 11 in the amounts of 0.4 and 0.3% of lysine and methionine respectively, the growth rate was not much better than on the basal diet alone; whereas when they were added to diet 21 in the smaller amounts of 0.13 and 0.15%, the rate of growth was greatly im-

proved. Russell and associates ('52), in testing the effects of a 200% excess of each essential amino acid in turn, when the others just met the rat requirements, found that only the excess of lysine or methionine (with the possible exception of valine) caused a significant repression in growth in rats. These authors concluded that the growth-repressing property is characteristic of lysine and methionine specifically, and is not due simply to an amino acid imbalance. However, the effect of simultaneous excesses of lysine and methionine in the diet of rats was not investigated.

TABLE 3

Estimated essential amino acids in the basal diets [1]

CONSTITUENT	DIET 11	DIET 21	RAT GROWTH REQUIREMENT [2]
	%	%	%
Lysine	0.62	0.56	1.0
Phenylalanine + tyrosine	1.02	1.12	0.9
Phenylalanine only	(0.50)	(0.52)	
Leucine	1.02	1.19	0.8
Valine	0.66	0.68	0.7
Methionine + cystine	0.34	0.37	0.6
Methionine only	(0.19)	(0.20)	
Isoleucine	0.53	0.53	0.5
Threonine	0.41	0.42	0.5
Histidine	0.28	0.34	0.4
Tryptophan	0.13	0.12	0.2
Arginine	0.47	0.46	0.2

[1] The estimations are based on the amino acid contents of the feed ingredients, as compiled by Sievert and Fairbanks ('55). The values for whole corn were also used for the amino acid contents of corn flakes.

[2] The rat requirements for growth are as suggested and modified by Rose and associates (Rose et al., '49; Wretlind and Rose, '50).

With diet 11, the addition of histidine in the presence of added lysine and methionine had but little effect on growth. With diet 21, the addition of 0.10% of DL-tryptophan with 0.15% of DL-methionine was able to overcome to a large extent the growth rate depression caused by methionine; but in the presence of added lysine and methionine, the added tryptophan seemed to have practically no effect on growth. Accord-

ing to Salmon ('53), the trytophan requirement of growing rats was only 0.13% of the diet when the protein content was only 10.8%, and adequate niacin was present. If a similar requirement holds for the growing dog, then the amounts of tryptophan in the basal diets may be enough, provided that the utilization of tryptophan is not decreased by a relative excess of some other amino acid.

Threonine may also have been somewhat limiting in the two basal diets, especially in view of the fact that it may not be available for utilization from some corn proteins (Geiger et al., '52; Sure, '53a) but the effect of the addition of threonine was not tested. Harper et al. ('54a) reported that under certain conditions a threonine deficiency can be induced in the rat by the addition of methionine to a low-protein diet.

Harper and associates ('54b, '55) found that an excess of dietary leucine can cause a growth retardation in rats, and that this effect can be almost entirely overcome by the addition of a small amount of isoleucine to the diet. Benton et al. ('55) reported that under certain conditions an addition of leucine had a growth-repressing effect, whereas the same amount of leucine had a slight stimulatory effect on growth when isoleucine was added simultaneously to the same diet. Likewise, Sure ('53b) found that threonine alone caused a slight depression in the rate of growth of rats on a diet containing milled wheat, whereas the same amount of threonine had a very stimulatory effect on growth when lysine was also added. Although additions of lysine and methionine were not both made individually to the same diet in the present study, each amino acid by itself did retard growth (on different diets), while small amounts of the two added together caused a marked increase in the rate of growth on diet 21.

The importance of amino acid proportions in the diet was discussed by Flodin ('53) and more recently by Elvehjem and Harper ('55). Harper et al. ('54b) have listed many references concerning growth retardations caused by excesses of various individual, common amino acids. These authors pointed out that it usually takes a smaller amount of an

effective amino acid to repress growth on a low-protein diet than is needed in order to achieve that effect on a diet containing more protein.

SUMMARY AND CONCLUSIONS

It is concluded that caution should be used in trying to improve low-protein diets for young, growing dogs by the additions of single amino acids. The average gains in weight of 8 or 9 pups per group for 10 or 12 weeks showed that an individual supplement of either L-lysine or DL-methionine caused a retardation in the growth which was already suboptimal. When both of these amino acids were added simultaneously, the growth repression caused by either alone was overcome by the combination, and in one instance the growth was greatly improved over that obtained with the basal diet alone. These observations are interpreted as showing evidence of the importance of amino acid balance in low-protein diets for growing dogs.

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