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Fewer calories same nutrients for woman 65 years

For the maintenance of health and well-being the recommended daily dietary allowances for "Women, 65 years," is lower in calories, yet is the same in essential nutrients as for younger age groups. A moderate reduction in dietary fat reduces the total calories. For those advising women in this age group about their diet, *a basic cereal and milk breakfast* as shown in the chart below merits consideration. Its fat content of 10.9 gm. provides 20 per cent of the total calories. *Thus it is a moderate low-fat breakfast.* As shown in the chart below, it provides for "Women, 65 years," and for younger age groups about one-fourth of the recommended daily dietary allowances of protein, important B vitamins, and essential minerals. The Iowa Breakfast Studies demonstrated that *a well-balanced, moderate low-fat basic cereal and milk breakfast was nutritionally efficient for the young and old alike.*

Recommended Daily Dietary Allowances and the Nutritional Contribution of a Basic Cereal and Milk Moderate Low-Fat Breakfast*

Menu: Orange Juice—4 oz.;
Cereal, dry weight—1 oz.;
Whole Milk—4 oz.; Sugar—1 teaspoon;
Toast (white, enriched)—2 slices;
Butter—5 gm. (about 1 teaspoon);
Nonfat Milk—8 oz.

Nutrients	Calories	Protein	Calcium	Iron	Vitamin A	Thiamine	Riboflavin	Niacin equiv.	Ascorbic Acid
Totals supplied by Basic Breakfast	503	20.9 gm.	0.532 gm.	2.7 mg.	588 I.U.	0.46 mg.	0.80 mg.	7.36 mg.	65.5 mg.
Recommended Dietary Allowances—Women, 65 Years (58 kg.—128 lb.)	1800	58 gm.	0.8 gm.	12 mg.	5000 I.U.	1.0 mg.	1.5 mg.	17 mg.	70 mg.
Percentage Contributed by Basic Breakfast	27.9%	36.0%	66.5%	22.5%	11.8%	46.0%	53.3%	43.3%	93.6%

Cereal Institute, Inc.: Breakfast Source Book. Chicago: Cereal Institute, Inc., 1959.
Food & Nutrition Bd.: Recommended Dietary Allowances, Revised 1958. Natl. Acad. Sci.—Natl. Research Council Publication 589, 1958.
Watt, B. K., and Merrill, A. L.: Composition of Foods—Raw, Processed, Prepared. U.S.D.A. Agriculture Handbook No. 8, 1950.

**The allowance levels are intended to cover individual variations among most normal persons as they live in the United States under usual environmental stresses. Calorie allowances apply to individuals usually engaged in moderate physical activity. For office workers or others in sedentary occupations they are excessive. Adjustments must be made for variations in body size, age, physical activity, and environmental temperature.*

Availability to Man of Amino Acids from Foods

III. THREONINE FROM CORN[1]

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Earlier papers have demonstrated that by using nitrogen balance as the criterion of comparison the availability to man of an amino acid in natural foods can be determined by feeding alternately an equivalent amount of a purified and of a bound amino acid in the food (Linkswiler et al., '58a; Linkswiler et al., '58b).

This paper reports the results of two studies conducted to determine the availability to man of threonine from corn.

EXPERIMENTAL

The experimental procedure was designed to measure the availability of threonine in corn by feeding in alternate periods a diet containing purified threonine and one containing the same amount of threonine from corn. Availability was measured from the nitrogen balance performance of the subjects. Two experiments were conducted which differed from each other only in that the diets used in study 1 provided 10 gm of nitrogen daily, whereas those in study 2 supplied 6 gm. Study 1 was of 40 days duration and consisted of a 20-day adjustment period and two 10-day test periods. Study 2 was 52 days in length and was divided into a 20-day adjustment period and two 16-day test periods. During each study a natural diet was fed during the first 5 days of the adjustment period and a semipurified diet, adequate in all known dietary essentials, for the remaining 15 days. The diets fed during the adjustment periods were equivalent in nitrogen content to the diets fed during the test periods and provided 620 mg of threonine daily, an amount which was double the minimum daily requirement for young women as reported by Leverton et al. ('56a). In study 1 all threonine was provided in purified form during the adjustment period; in study 2 during part of the adjustment period the threonine was provided partially from corn and partly in purified form. In each study the subjects were given 280 mg of threonine daily during the first test period and 195 mg for the second test period. During half of each test period the subjects received purified threonine, and during the other half threonine was supplied by corn. Part of the subjects received threonine from corn during the first half of each test period and purified threonine during the latter half while the remaining subjects received the diets in reverse order.

Although the primary purpose of these studies was to determine the availability of threonine from corn, the experimental design was suitable for determining requirement of the amino acid. Nitrogen balance data obtained on the various intakes of purified threonine may be compared with the results obtained from requirement studies by other workers.

Diets. The basal diet consisted of a few fruits low in nitrogen, cornstarch, sucrose, butterfat, vegetable fat, jelly, carbonated beverage, amino acids, additional sources of nitrogen and vitamin and mineral supplements (table 1). The amino acid composition of the diets is given in table 2. With the exception of threonine which was given at three levels, all essential amino acids were given in amounts approximately

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[1] This study was part of a North Central Regional Project (NC-49, Factors affecting requirements of adult human subjects for protein and amino acids), a cooperative study involving agricultural experiment stations in the North Central Region and supported in part by regional funds. Published with the approval of the Director as Paper no. 1044, Journal Series, Nebraska Agricultural Experiment Station.

[2] Present address: School of Home Economics, University of Wisconsin, Madison.

TABLE 1
Composition of basal diet

	10-gm Nitrogen diet	6-gm Nitrogen diet
	gm	*gm*
Orange juice	100	100
Applesauce	100	100
Peaches or pears	100	100
Baking powder[1]	7.6	7.6
Mineral mix[2]	3.6	3.6
Glycine	18.0	7.4
Diammonium citrate	27.0	7.5
Glutamic acid	19.2	11.3
Amino acid mix	9.1	9.1
Wafers[3]	One batch	One batch
Butterfat[4]		
Jelly[4]		
Sugar[4]		
Carbonated beverage[4]		
Coffee[5]		
B-complex vitamins[6]	One capsule	One capsule
Vitamins A and D[7]	One capsule	One capsule

[1] Recipe for daily amount: 2.05 gm $NaHCO_3$, 2.86 gm $Ca(H_2PO_4)_2 \cdot H_2O$; 2.69 gm cornstarch.
[2] The mineral supplement of Leverton et al. ('56a) was used.
[3] Recipe for daily amount (in grams): cornstarch, 100; sugar, 40; Mucilose flakes, 4; mineral mix, 3.6; baking powder, 7.6; fat, 60; NaCl, 2.0; and water, 60 ml.
[4] Amount varied, depending on the caloric requirement of individual.
[5] As desired.
[6] Each capsule contained the following (in milligrams): thiamine, 3.0; riboflavin, 2.5; nicotinamide, 20.0; pyridoxine, 1.0; pantothenic acid, 10.0; ascorbic acid, 50; and vitamin B_{12}, 20 μg; folic acid, 100 μg.
[7] Each capsule contained 5000 I.U. of vitamin A and 500 I.U. of vitamin D.

TABLE 2
Composition of the amino acid mix

Amino acids	Basal diet	90-gm Corn diet		60-gm Corn diet	
	Purified amino acids	Amino acids in corn	Purified amino acids	Amino acids in corn	Purified amino acids
	gm/person/day	*gm/person/day*		*gm/person/day*	
L-Arginine·HCl	0.992	0.455	0.537	0.303	0.689
L-Histidine·HCl	0.492	0.243	0.249	0.162	0.330
L-Lysine·HCl	1.249	0.228	1.021	0.152	1.097
L-Methionine	0.460	0.178	0.282	0.119	0.341
L-Tryptophan	0.320	0.042	0.278	0.028	0.292
L-Phenylalanine	0.440	0.335	0.105	0.223	0.217
L-Threonine	—[1]	0.260	0.000	0.175	0.000
L-Leucine	1.240	1.125	0.115	0.750	0.490
L-Isoleucine	0.627[2]	0.328	0.299	0.218	0.409
L-Valine	1.300	0.405	0.895	0.270	1.030
L-Cystine	0.450	0.132	0.318	0.088	0.362
L-Tyrosine	0.900	0.516	0.384	0.344	0.556

[1] Variable, 600 to 175 mg.
[2] Given in DL form for study 1; L form for study 2.

double those reported to be the minimum daily requirements for young women (Leverton et al., '56a, b, c, d, e; Swendseid et al., '56; Swendseid and Dunn, '56; Jones et al., '55,[3] '56). Arginine, histidine, cystine and tyrosine were also given. Glycine, glutamic acid and diammonium citrate were added to raise the total nitrogen intake to either 6 or 10 gm daily, glycine and diammonium citrate each furnishing 40% and glutamic acid, 20% of the nitrogen provided by these three compounds.

[3] Jones, E. M., C. A. Baumann and M. S. Reynolds 1955 Methionine and lysine requirement of mature women. Federation Proc., *14:* 438 (abstract).

The corn diet was also semipurified in nature. The main difference between the two diets was that the 12 amino acids in the corn, as determined by microbiological assay in this laboratory, replaced equal amounts of purified amino acids, thus keeping constant the intake of the 12 amino acids (table 2). During the test periods when the corn diet was fed, all the threonine came from corn except the 20 mg present in the low nitrogen foods which were constant throughout the entire experimental period. Corn was fed in 90 and 60 gm amounts, 90 gm of corn providing 1.25 gm of nitrogen and 260 mg of threonine, and 60 gm, 0.84 gm nitrogen and 175 mg threonine. Degerminated, ground white corn was used. Sufficient water was added to the cornmeal, and the mixture was steamed for 40 minutes and served as a cereal.

The amino acid content of the corn and of the low-nitrogen foods was determined microbiologically (Steele et al., '49). Both *Leuconostoc mesenteroides* and *Leuconostoc citrovorum* were used as test organisms for the threonine determinations, and the respective values obtained were 2.85 and 2.93 mg per gm of corn. Recoveries were 98 and 101%.

The amount of calories required for each subject to maintain weight was determined during the adjustment period of each study, and this amount was maintained constant during the test periods.

Subjects. Ten college women ranging in age from 18 to 27 years served as subjects for study 1, and 7 college women and one man ranging in age from 17 to 42 years were subjects for study 2. Pertinent information concerning the subjects is given in table 3.

RESULTS

The individual and mean nitrogen balances of the subjects of study 1 (10 gm nitrogen intake) during the last 5 days of the adjustment period and the two 10-day test periods are presented in figure 1. The threonine intake included 20 mg which was present in the low nitrogen foods. With an intake of 620 mg of threonine the mean nitrogen balance of all subjects was 0.05 gm daily. When the threonine intake was reduced to 280 mg, the mean nitrogen balance of the subjects was −0.25 gm daily using purified threonine and −0.27 gm using the corn threonine. With an intake of 195 mg of threonine the mean nitrogen

TABLE 3

Vital statistics and the caloric intakes of each subject[1]

Subject no.	Age	Weight		Height	Average Cal./day
		Initial	Final		
	years	*kg*	*kg*	*cm*	
		Study 1			
30	21	50.2	48.1	159.4	2025
31	23	54.3	53.6	157.5	2192
32	20	52.5	52.0	156.8	2055
34	19	61.4	61.3	163.2	2427
35	18	60.5	60.5	168.3	2447
36	18	62.4	62.9	167.6	2408
37	19	62.0	62.1	164.5	2351
38	27	58.9	57.8	158.7	2285
39	21	57.8	56.3	168.9	2317
40	20	60.5	58.7	168.9	2309
		Study 2			
42	20	52.4	53.1	156.8	2191
43	38	80.5	82.0	177.2	3875
44	42	69.5	70.0	155.6	2295
45	20	62.8	62.0	165.1	2427
46	17	66.5	66.0	167.6	2751
47	17	56.0	54.9	166.4	2473
48	20	59.8	59.3	161.3	2316
49	17	81.8	81.5	183.5	3649

[1] All subjects were female adults except no. 43, a male adult.

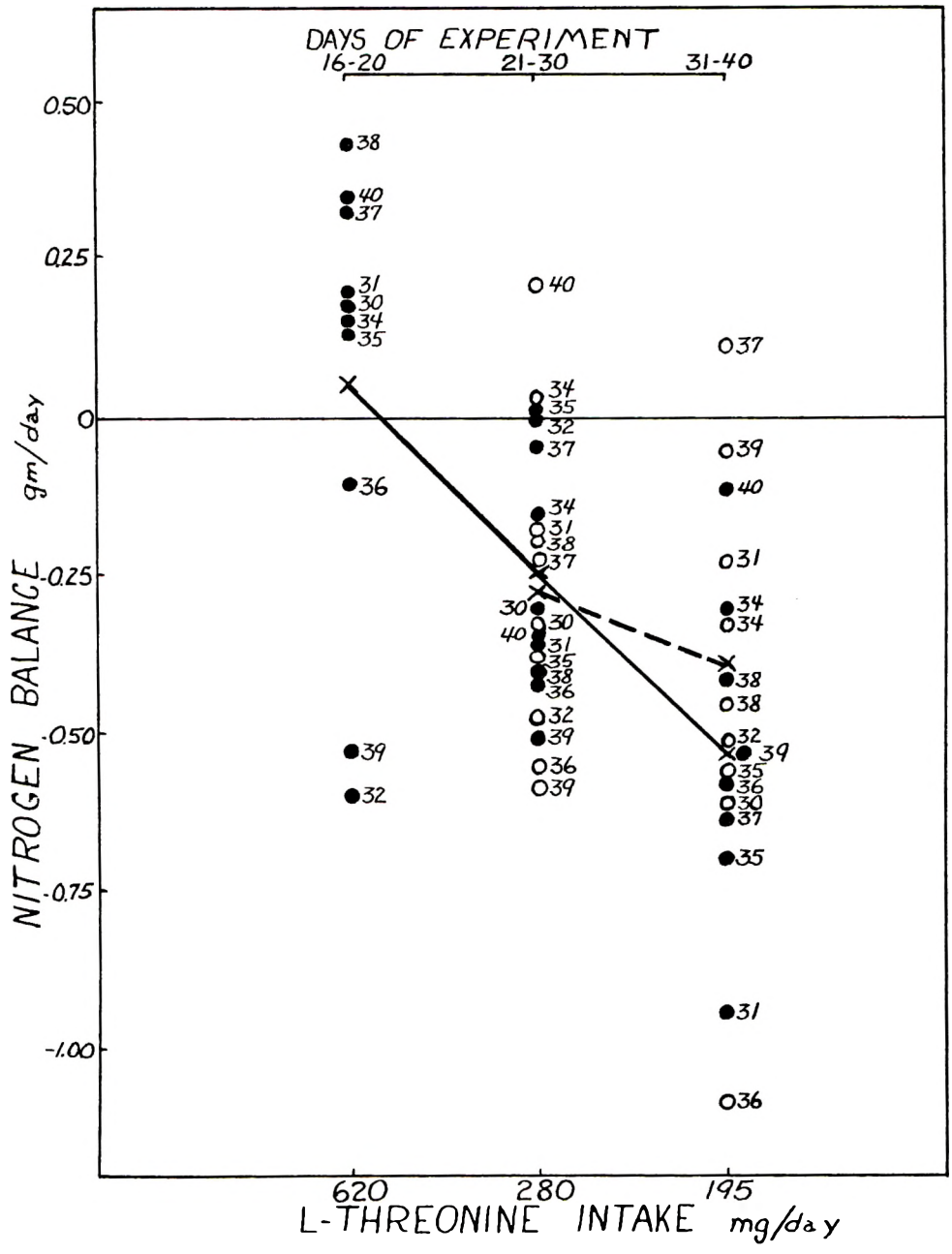

Fig. 1 Individual and mean nitrogen balances of 10 subjects receiving varying intakes of threonine, 10 gm of nitrogen per day. The numbers in the body of the graph refer to subjects. Individual nitrogen balances when the threonine was supplied by corn, ○; or by purified threonine, ●. Curve ———, mean daily nitrogen balance for all subjects when threonine was supplied by corn. Curve ———, mean daily nitrogen balance for all subjects when purified threonine was given.

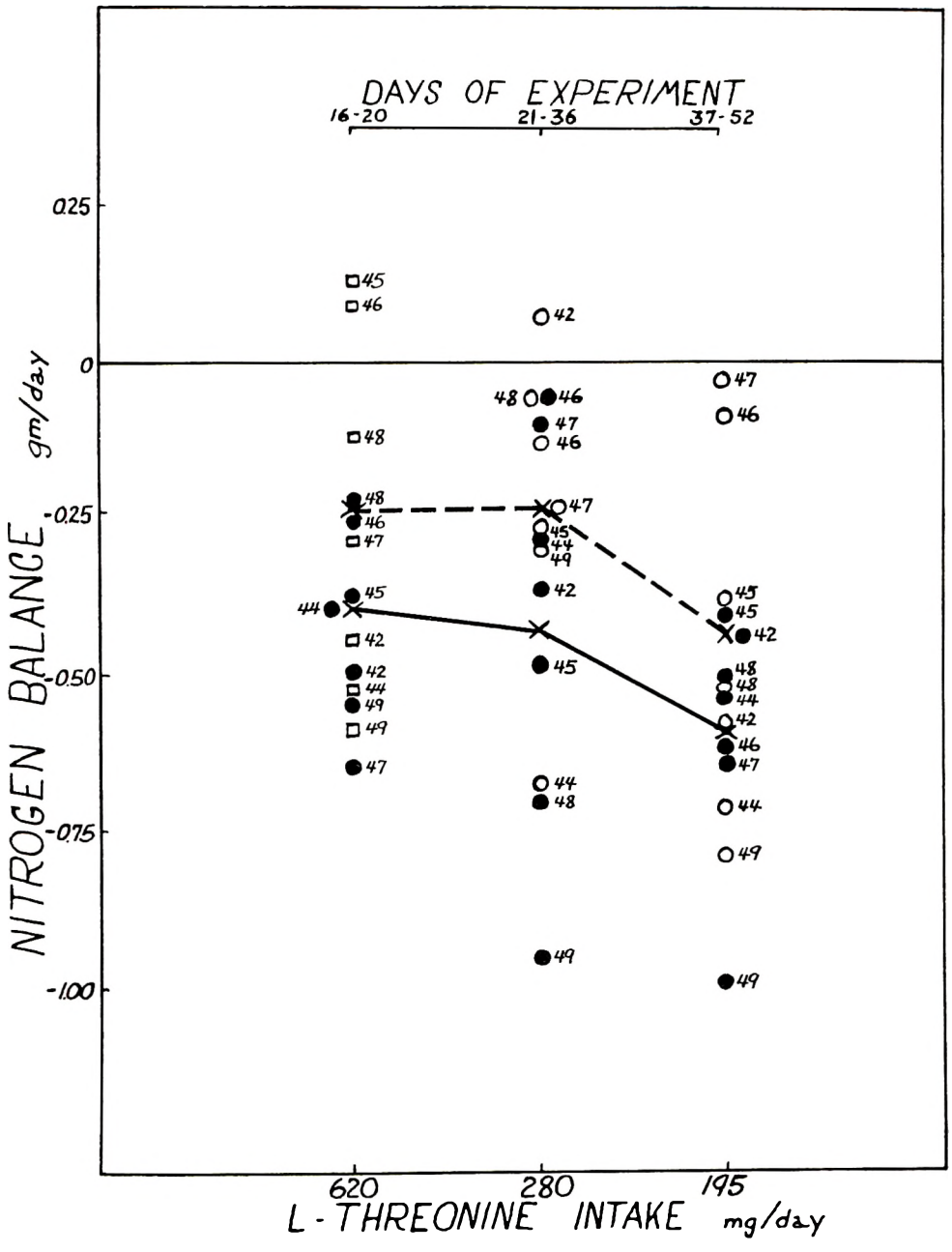

Fig. 2 Individual and mean nitrogen balances of 7 subjects receiving varying intakes of threonine, 6 gm of nitrogen per day. The numbers in the body of the graph refer to subjects. Individual nitrogen balances when threonine was supplied by a combination of purified and corn threonine, □; by corn, ○; or by purified threonine, ●. Curve – – – –, mean daily nitrogen balance for all subjects when threonine was supplied by corn, except for days 16 to 20 when a combination of purified and corn threonine was given. Curve ———, mean daily nitrogen balance for all subjects when purified threonine was given.

balance using purified threonine was −0.53 and with corn threonine, −0.42 gm.

Figure 2 shows the individual and mean nitrogen balances of the women subjects in study 2 (6 gm of nitrogen daily) during the last 10 days of the adjustment period and the two test periods. The mean nitrogen balance using purified threonine with an intake of 620 mg was −0.40 gm daily. When the subjects were given 620 mg of threonine, 260 of which was supplied by 90 gm of corn and the remainder by purified threonine and that present in the low nitrogen foods, the mean nitrogen balance was −0.25 gm daily. Nitrogen balance was not affected when the threonine intake was reduced to 280 mg daily, the mean nitrogen balance being −0.43 using the purified threonine and −0.23 with the corn threonine. Further reducing the threonine intake to 195 mg resulted in a nitrogen loss of 0.59 gm using purified threonine and 0.44 gm with corn threonine.

Subjects 30, 32 and 40 vomited on several occasions during the last period of the 195 mg threonine intake. Consequently, nitrogen balance data were incomplete for this period and are excluded from the graph.

All nitrogen balance data of the male subject were omitted from the graph. For this subject the respective nitrogen balances observed when receiving corn and purified threonine were −1.04, −0.32 (620 mg threonine); −0.34, −1.45 (280 mg threonine); −1.70, −1.50 (175 mg threonine).

While receiving 195 mg threonine, the subjects of study 1 who were given 10 gm of nitrogen complained of nausea and vomiting, but the subjects on study 2 who were receiving an intake of 6 gm nitrogen did not exhibit these symptoms.

The mean nitrogen balances of the subjects were usually higher when they received threonine from corn than when they were given purified threonine. These differences are not statistically significant; for study 1, "t" = 0.198 at the 280-mg level and 1.140 at the 195-mg level; for study 2, "t" = 1.259 at the 280-mg level and 1.300 at the 195-mg level.

The subjects of study 1 consumed an average of 39 Cal. per kg of weight and those of study 2, 40 Cal. These caloric intakes permitted the subjects to maintain weight.

DISCUSSION

Results reported herein and those from a previous publication (Linkswiler et al., '58b) indicate that the amino acids, valine and threonine in corn are completely available to adult man. On the other hand, reports from many laboratories have indicated impaired availability of essential amino acids from certain foods.

That nitrogen was a limiting nutrient when fed at a level of 6 gm daily is indicated since the subjects were in negative balance on an intake of 620 mg of threonine, an amount which resulted in a positive nitrogen balance for subjects consuming 10 gm of nitrogen. Decreasing the threonine intake from 620 to 280 and finally to 195 mg resulted in a progressively larger nitrogen loss for those on the higher nitrogen intake. A comparable reduction in threonine intake had no effect on nitrogen balance for subjects consuming 6 gm of nitrogen until the threonine level was reduced to 195 mg daily, thus suggesting that nitrogen was more limiting than threonine with the two highest intakes of the amino acid. No attempt was made to establish nitrogen equilibrium by increasing caloric intake. As has been the practice in previous studies of this series, calories were given in amounts which allowed each subject to maintain weight.

In the present study subjects receiving 6 gm of nitrogen and the lowest level of threonine (195 mg) experienced no subjective symptoms, whereas those fed the same amount of threonine and 10 gm of nitrogen experienced frequent nausea and vomiting. Even though nitrogen balances were more favorable among subjects on the higher nitrogen intake, the occurrence of these symptoms suggests that these subjects may have been more deficient in threonine than those receiving the lower level of nitrogen. Leverton et al. ('56a) reported that the level of dietary nitrogen may influence the threonine requirement of young women.

The severe nitrogen losses of the male subject suggest that the essential amino acid mixture as well as total nitrogen may have been inadequate. Amounts of methionine and isoleucine provided were slightly

less that the minimal requirements established by Rose ('55). Calories appeared to be not limiting since the subject tended to gain weight with an intake of 46 Cal. per kg of body weight.

Arbitrary criteria have been used for judging the adequacy of amino acid intake. It appears that in each case the aim has been to determine the least amount of an amino acid which permits nitrogen equilibrium according to the definition of the particular investigator. Perhaps a more valid criterion would be to determine the amount of an amino acid which permits optimal performance in nitrogen balance with the particular experimental diet being used. The graphs presented in this paper as well as in an earlier paper of this series (Linkswiler et al., '58b) show a definite downward trend in the slope of the line connecting the mean nitrogen balances of the subjects as the intake of an amino acid is lowered. The relationship between nitrogen balance and amino acid intake observed in these studies is comparable with the growth response of animals when graded amounts of a specific nutrient are fed. In the latter case, adequacy is judged as the amount of nutrient beyond which increments in intake give no further growth response. It, therefore, seems logical to apply the same reasoning to nitrogen balance experiments in assessing amino acid requirements. It is certainly obvious that an intake of 280 mg of threonine daily is less satisfactory for the subjects of this experiment than an intake of 620 mg. Furthermore, it appears that the requirement of these young women may exceed 310 mg, the amount reported as being the minimum daily requirement of young women (Leverton et al., '56a).

Erratic behavior in nitrogen balance makes it difficult to assess the amino acid requirement for an individual. For example, subject 39 showed a more favorable nitrogen balance when receiving one of the diets providing 195 mg of threonine than with any of the diets containing higher amounts of the amino acid. Observations such as this suggest the desirability of assessing amino acid requirement from the mean nitrogen balance of a group of subjects rather than attempting to estimate the need of a particular individual, even though requirements may vary somewhat from one individual to another.

SUMMARY

Two studies were conducted to determine the availability to human subjects of threonine in corn. The only difference in the diets between the two studies was that one contained 10 gm of nitrogen daily and the other, 6 gm. Availability was determined by feeding in alternate experimental periods equivalent amounts of purified threonine and corn threonine and comparing the resultant nitrogen balance. In both studies threonine in corn was completely available to man.

The mean nitrogen balance of the subjects consuming the diet containing 10 gm of nitrogen and 620 mg of threonine was slightly positive, but that of subjects receiving 6 gm of nitrogen and 620 mg of threonine was distinctly negative. Failure to achieve nitrogen equilibrium with the lower nitrogen intake may be attributed to an insufficiency of nitrogen and/or calories. The threonine requirement for young women appears to exceed 280 mg per day.

ACKNOWLEDGMENT

The authors are grateful to the students who served as subjects in this study, to Mrs. Donna Geschwender and Mrs. Shirley Chen for technical assistance and to Mrs. Nancy Byerly for assistance as dietitian.

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Availability to Man of Amino Acids from Foods

IV. ISOLEUCINE FROM CORN[1]

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Earlier papers have presented a method suitable for determining the availability to man of amino acids from foods (Linkswiler et al., '58a) and have shown that valine (Linkswiler et al., '58b) and threonine (Linkswiler et al., '60) from corn are completely available to the adult man.

The present paper is a continuation of the series and is concerned with determining the availability of isoleucine in corn.

EXPERIMENTAL

Procedure. The experimental procedure followed was essentially that reported in an earlier paper (Linkswiler et al., '60). The experiment was 32 days in length. A pre-experimental period of three days was followed by a 7-day adjustment period and two test periods, one 10 days in length and the other, 7 days. Because isoleucine was low enough during the last test period to produce deficiency symptoms, the intake of this amino acid was increased and the subjects were studied an additional 5 days. The subjects were divided into two groups; one was given a diet providing 6 gm of total nitrogen daily and the other, a diet containing 10 gm. The corn diet was fed during the 7-day adjustment period. It was supplemented with purified isoleucine to provide a total of 922 mg daily. During the first test period 422 mg of isoleucine were given and during the second, 222 mg. The sequence in which the subjects received the amino acid from corn and the purified amino acid has been described (Linkswiler et al., '60).

Diet. Because subjects tend to go into a markedly negative nitrogen balance when they are transferred from a diet of natural foods to a semipurified diet of equivalent nitrogen content, they were given a pre-experimental depletion diet which contained 2.7 gm of nitrogen and consisted of bread, fruit, leafy vegetables and sufficient sugar and fat to satisfy caloric needs. This diet was fed in order to bring the subjects to nitrogen equilibrium more quickly. Hegsted et al. ('46) reported that it is impossible to reach a stabilized nitrogen output in a short time unless a preliminary very low-nitrogen period is used. The basal (purified amino acid) diet was the same as that given earlier (Linkswiler et al., '60) with two exceptions: (1) the threonine intake was maintained constant at 600 mg while the isoleucine content was varied from 922 to 222 mg; (2) all amino acids were given as L-isomers throughout the study. The corn diet differed from the basal diet in that the 12 amino acids present in corn replaced equal amounts of the purified amino acids. The amounts of corn given for the two test periods were 132 and 66 gm which contained 1.71 and 0.85 gm of nitrogen and 400 and 200 mg of isoleucine. The amount of calories required by each subject to maintain body weight was determined during the adjustment period, but the caloric intake of individual subjects was maintained constant during the test periods.

The amino acid content of the corn and of the low-nitrogen foods was determined microbiologically (Steele et al., '49). Isoleucine was determined using *Leuconostoc mesenteroides* and *Leuconostoc citrovorum* as test organisms, with respective values

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[1] This study was part of a North Central Regional Project (NC-49, Factors affecting requirements of adult human subjects for protein and amino acids), a cooperative study involving agricultural experiment stations in the North Central Region and supported in part by regional funds. Published with the approval of the Director as Paper no. 1045, Journal Series, Nebraska Agricultural Experiment Station.

[2] Present address: School of Home Economics, University of Wisconsin, Madison.

TABLE 1

Vital statistics and the caloric and nitrogen intakes of each subject

Subject no.	Age	Sex	Weight		Height	Caloric intake	Total nitrogen intake
			Initial	Final			
	years		*kg*	*kg*	*cm*		*gm/day*
70	30	F	52.7	51.9	154.3	2145	6
71	27	F	46.6	47.9	150.5	2003	6
73	19	F	47.3	47.7	157.5	2253	6
74	18	M	54.8	55.2	170.2	2788	6
75	21	F	58.6	59.2	165.1	2298	6
76	29	F	55.5	56.1	160.0	2359	10
77	30	F	52.3	52.8	160.0	2107	10
78	26	F	65.0	65.7	163.2	2489	10
79	22	F	65.5	65.5	166.4	2447	10
80	23	M	75.0	73.9	175.9	3091	10
81	21	M	80.9	81.3	174.0	3235	10

for corn being 2.99 and 3.03 mg of isoleucine per gram. Recoveries were 98 and 101%.

Subjects. Three young men and 8 young women participated in the experiment. Pertinent information concerning the subjects is given in table 1.

RESULTS

Subjects reached a stabilized nitrogen output rapidly on the semipurified diets following three days on the pre-experimental depletion diet. Individual and mean daily nitrogen balances of 4 young women receiving 4 levels of isoleucine and 10 gm of nitrogen are presented in figure 1. The low nitrogen foods present in the experimental diets provided 22 mg of isoleucine, and this amount has been included in the total isoleucine content of each diet. During the last 5 days of the adjustment period the mean nitrogen balance of the 4 subjects was 0.37 gm, with an intake of 922 mg of isoleucine. When the isoleucine intake was reduced to 422 mg, the mean nitrogen balance of subjects receiving isoleucine from corn was 0.06 gm daily and from purified isoleucine, − 0.35 gm. Further reduction of the isoleucine intake to 222 mg resulted in mean nitrogen balances of − 0.27 gm using corn isoleucine and − 0.63 gm when receiving purified isoleucine.

Data for the two men have been omitted from the graph and are presented in table 2. Unlike the women, the men were unable to attain nitrogen equilibrium when receiving the highest isoleucine intake. Negative nitrogen balances persisted throughout the study. Increasing the isoleucine (day 28) and the subsequent provision of larger quantities of the other essential amino acids (days 29 to 32) improved nitrogen balance. In the short period of observation it was not possible to discern whether the improvement was due to the increase in isoleucine or to the overall increase in essential amino acids.

Figure 2 presents the individual and mean daily nitrogen balances of the 4 young women and one young man given 6 gm of nitrogen daily. The data obtained from this man are included with those of the women because there was no obvious difference between his nitrogen balance performance and that of the women subjects. The mean nitrogen balance with an intake of 922 mg of isoleucine was 0.07 gm daily. When the isoleucine intake was reduced to 422 mg, the mean nitrogen balance of subjects receiving isoleucine from corn was − 0.04 gm while that of subjects fed purified isoleucine was − 0.49 gm. When the isoleucine intake was further reduced to 222 mg, the respective values for subjects receiving corn and purified isoleucine were − 0.63 and − 0.76 gm daily.

After the subjects had received 222 mg of isoleucine for a few days, those fed both levels of nitrogen seemed very depressed and complained of being tired. The severity and complexity of the symptoms increased rapidly so that it was necessary to increase the intake of isoleucine after 7 days. Symptoms and complaints were: persistent headaches which did not re-

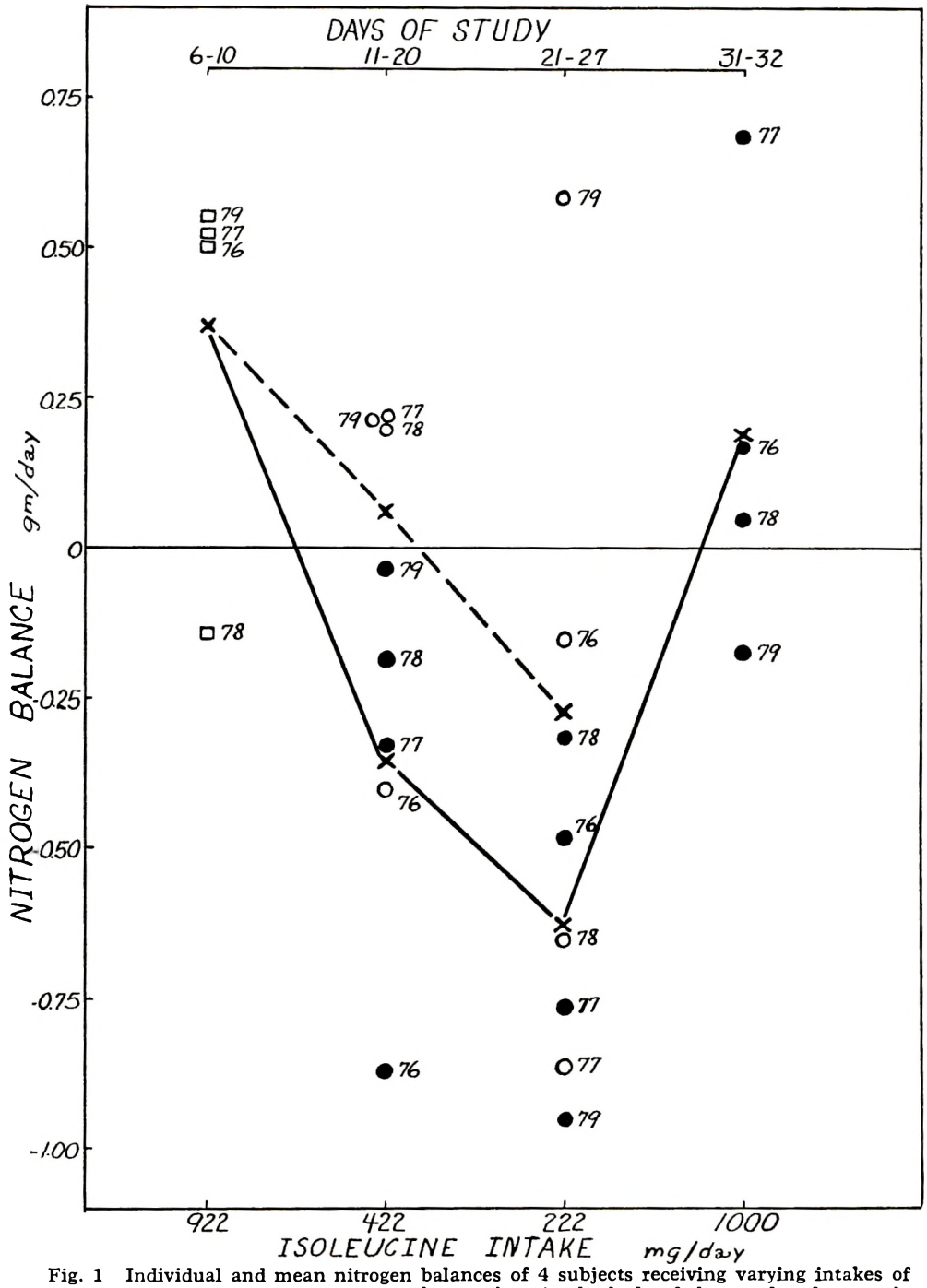

Fig. 1 Individual and mean nitrogen balances of 4 subjects receiving varying intakes of isoleucine, 10 gm of nitrogen per day. The numbers in the body of the graph refer to subjects. Individual nitrogen balances when isoleucine was supplied by a combination of purified and corn isoleucine, □; by corn, ○; or by purified isoleucine, ●. Curve – – – –, mean daily nitrogen balance for all subjects when isoleucine was supplied by corn, except for days 6 to 10 when a combination of purified and corn isoleucine was given. Curve ——, mean daily nitrogen balance for all subjects when purified isoleucine was given.

TABLE 2

Mean daily nitrogen balances of two men receiving diets containing 10 gm of nitrogen and varying quantities of isoleucine

Days	Amount of isoleucine	Source of[1] isoleucine	Other dietary modifications	Mean nitrogen balance	
				Subject no. 80	Subject no. 81
	mg			*gm*	*gm*
6 to 10	922	Corn, 400 mg Purified, 500 mg	—	−0.46	−1.09
11 to 15	422	Purified	—	−1.06	−0.77
16 to 20	422	Corn	—	−0.73	−0.79
21 to 25	222	Purified	—	−1.06	−0.82
26 to 27	222	Corn	—	−0.83	−1.93
28	1022	Corn, 200 mg Purified, 800 mg	—	−1.10	−0.30
29 to 30	1372	Corn, 600 mg Purified, 750 mg	2 times amount of other essential amino acids	−0.10	−0.13
31 to 32	722	Purified	2 times amount of other essential amino acids	−0.10	−0.16

[1] An additional 22 mg of isoleucine was available from the basal diet.

spond to medication with aspirin; sore, dry throats which were inflamed, almost hemorrhagic in appearance; unusual thirst; blood in feces; physical exhaustion; "bad" dreams; and mental depression. All subjective symptoms disappeared within 5 days after the subjects were given additional isoleucine, and the mean nitrogen balance became positive.

The difference between the performance of subjects fed corn and purified isoleucine is statistically significant at the 422-mg intake: at 10 gm of nitrogen, "t" = 6.147; at 6 gm of nitrogen, "t" = 7.109. The difference is not statistically significant at the 222-mg intake: at 10 gm of nitrogen, "t" = 0.862; at 6 gm of nitrogen, "t" = 0.941.

The women subjects fed 6 gm of nitrogen consumed 43 Cal. per kg of body weight and the man, 51 Cal. per kg. At the 10-gm nitrogen level the women consumed 40 Cal. per kg of body weight and the men, 41 Cal. per kg.

DISCUSSION

Isoleucine in corn appears to be completely available to human subjects. These findings are in agreement with those previously reported for valine and threonine (Linkswiler et al., '58b; Linkswiler et al., '60). In contrast, isoleucine has been reported to be only partially available to the rat (Elvehjem, '56; Benton et al., '55). Deshpande et al. ('57) reported that isoleucine in zein was only 30% available. There is a possibilty that the difference between the results obtained in this laboratory with human subjects and those obtained in other laboratories using rats is due to a species difference. It should be noted, however, that there are important differences in methodology. The method used in most animal studies has been to add to a basal diet increments of an amino acid and to compare the growth response with that obtained when increments of the amino acid were added by an intact protein. This, of course, results in a higher protein content for the diets containing the intact protein. Reports that the requirement for an amino acid is increased as the protein level is increased (Grau, '48; Almquist and Merritt, '50; Brinegar et al., '50; Grau and Kamei, '50; Salmon, '54; Griminger et al., '56; Becker et al., '57) raise the question as to the validity of this method for assessing availability of an amino acid. In this series of studies with human subjects the main difference between the basal or control diet and the corn diet is that the 8 essential amino acids and cystine, tyrosine, arginine and histidine in the corn replaced equal amounts of purified amino acids, thus keeping constant the intake of the 12 amino acids in the two diets. Using rats, Schwartz et al. ('59)

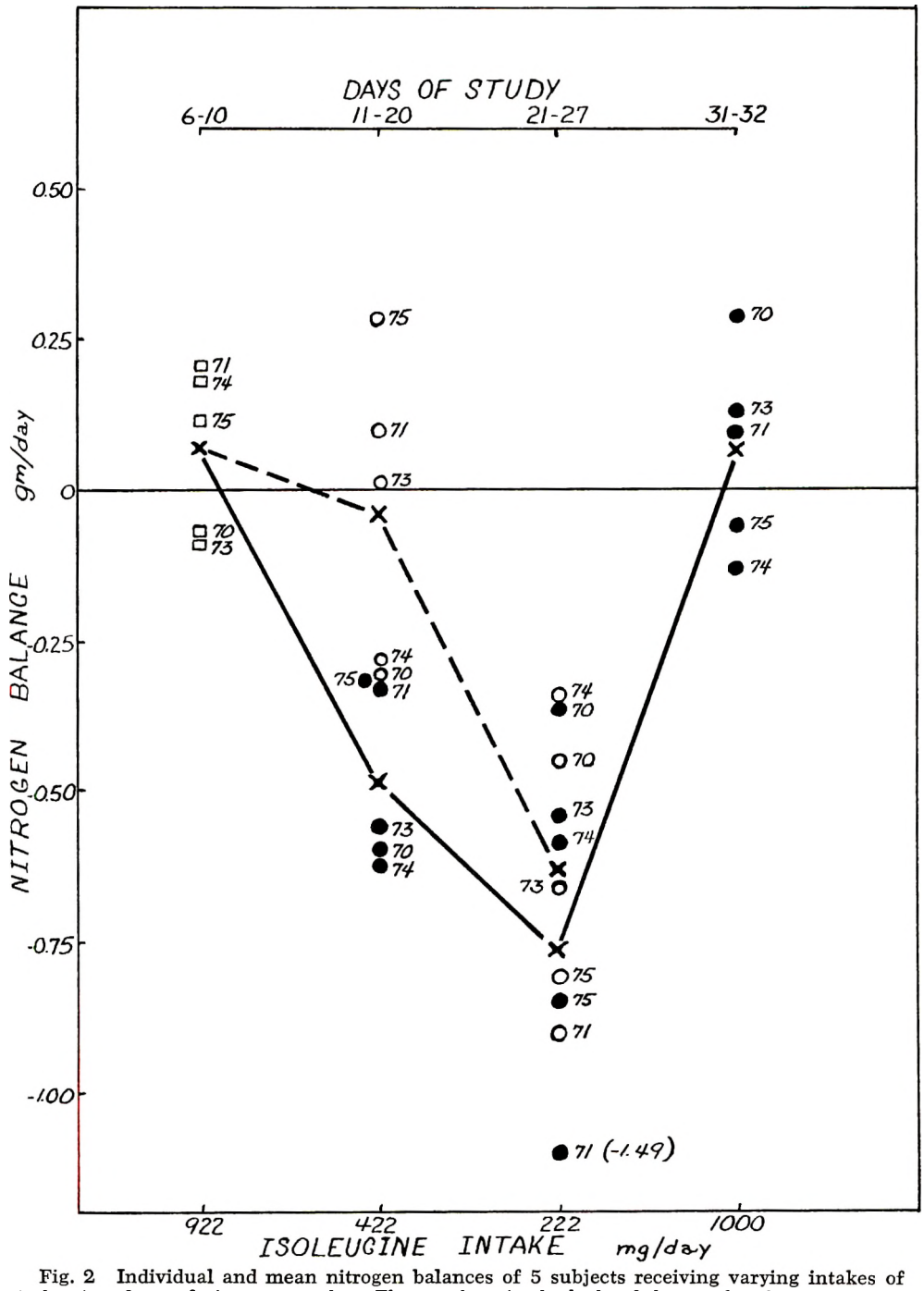

Fig. 2 Individual and mean nitrogen balances of 5 subjects receiving varying intakes of isoleucine, 6 gm of nitrogen per day. The numbers in the body of the graph refer to subjects. Individual nitrogen balances when isoleucine was supplied by a combination of purified and corn isoleucine, □; by corn, ○; or by purified isoleucine, Curve – – –, mean daily nitrogen balance for all subjects when isoleucine was supplied by corn, except for days 6 to 10 when a combination of purified and corn isoleucine was given. Curve ———, mean daily nitrogen balance for all subjects when purified isoleucine was given.

conducted lysine availability studies in which both methods, essentially as described, were used. When the test food replaced part of the protein of the basal diet so that the protein content of the two diets was the same, rats grew equally well with either diet; this was not true when the test food was added to the basal diet.

Usually slight improvements in nitrogen retention by subjects have been observed in this laboratory, using the diets containing the intact protein. In the preceding papers of this series these differences were not statistically significant. The significantly better nitrogen retention of the subjects while receiving the corn isoleucine indicates that this amino acid in the intact protein may be more efficiently utilized than the free amino acid.

The data presented in this paper indicate that the isoleucine requirement of these women is greater than 422 mg daily, particularly when the purified amino acid is given. This is true regardless of whether the criterion of Rose et al. ('54), that of Leverton et al. ('56) or that suggested by Linkswiler et al. ('60) is used as a means of assessing amino acid requirement. Swendseid and Dunn ('56) reported that the isoleucine requirement of 7 young women varied from 250 to 450 mg. Rose et al. ('55) found that the isoleucine requirement of 4 men was remarkably constant, varying only from 650 to 700 mg per day.

Other investigators have reported profound disturbances in human subjects when the isoleucine intake was restricted (Rose et al., '55; Swendseid and Dunn, '56). The symptoms observed during this study and those reported by Swendseid and Dunn indicate that the mucous membranes are involved. The rapidity with which an isoleucine deficiency develops and the severe and complex nature of the symptoms merit a study of the underlying metabolic causes. It should be noted that the lowest isoleucine intake in this study was considerably more (222 mg) than the lowest intake reported by Swendseid and Dunn (50 mg) and Rose and co-workers (0). It is evident that the symptoms described above were due to a deficiency of isoleucine since all symptoms disappeared and the subjects experienced a sense of well-being within 5 days after administration of 1000 mg of isoleucine per day. It has been reported that chicks fed diets devoid of isoleucine survived less than 20 days and exhibited signs of extreme physical distress (Ousterhaut, '60). Ousterhaut reported that the level of free isoleucine in the tissues of these chicks decreased as a result of isoleucine deprivation and suggested that the apparent extreme discomfort and early deaths were due to an imbalance of free amino acids in the tissues.

SUMMARY

The availability of isoleucine in corn for human subjects has been determined using experimental diets containing 6 or 10 gm of nitrogen. According to the nitrogen balance data obtained isoleucine in corn seems to be completely available. With an intake of 422 mg of isoleucine daily, nitrogen retention was significantly better in response to isoleucine from corn than to purified isoleucine. The isoleucine requirement appears to be greater than 422 mg per day for women as well as for men. Symptoms of isoleucine deficiency developed within 7 days with a daily intake of 222 mg.

ACKNOWLEDGMENT

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Amino Acid Reference Patterns:

A COMPARISON OF THE PATTERN OF HUMAN MILK WITH THE FAO PATTERN IN HUMAN NUTRITION[1]

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In 1955, a Committee of the Food and Agricultural Organization of the United Nations (FAO) described a "provisional" pattern of amino acids which would help in evaluating the protein quality of individual foods (FAO, '57). The figures were derived from the existing information of the amino acid requirements of adult men and women and of infants who seemed to require similar proportions of the essential amino acids. This pattern was formulated to take into consideration a proper balance between the amino acids as well as meeting individual amino acid requirements. The committee noted that the patterns of high quality proteins such as milk or egg were very similar to the provisional pattern except that the natural foods contained considerably more leucine.

The present study was designed to determine whether a feeding formulated according to the FAO pattern would have any advantages or disadvantages when compared with a natural feeding, human milk. In order to control conditions as well as possible, a completely synthetic diet was used, the protein moiety of which was a mixture of 18 L-amino acids. Our subjects were premature infants who, because of their rapid rate of growth, might be most sensitive to differences in protein quality. The diets contained 2 gm of protein per kg of body weight. This low level of protein was chosen to exaggerate any differences between the two feedings.

METHODS

The composition of the two experimental diets was the same except for the ratio of the individual amino acids; in one it was similar to human milk while the other contained the essential amino acids in the pattern designed by the FAO. In the second diet, the unessential amino acids were provided in the human milk pattern since the FAO was concerned only with the ratio of the essential amino acids. Table 1 shows the composition of the experimental diet. In table 2 are listed the amino acid mixtures.

TABLE 1
Composition of experimental diet

	gm	*% of total calories*
L-Amino acid mixture	100	5.5
Corn oil[1]	290	39
Maltose and dextrins[2]	1000	55.5
Mineral mixture[3]	48.50	
	ml/day	
B-vitamin mixture[4]	10	
Vitamins[5] A, C and D	0.6	

[1] Mazola, supplied by the Corn Products Refining Company, New York.
[2] Dextri-Maltose, supplied by Mead Johnson and Company.
[3] Percentage composition of the mineral mixture: NaCl, 18.9; $CaHPO_4$ (anhydrous), 25.4; $MgSO_4$ (anhydrous), 6.8; $KHCO_3$, 44.4; KCl, 2.88; Fe_3 citrate, 2.21; $CuSO_4$ (anhydrous), 0.24; $MnSO_4$ (anhydrous), 0.15; KI, 0.015; NaF, 0.03.
[4] The B-vitamin mixture provided the following daily (in milligrams): thiamine·HCl, 0.38; riboflavin, 2.0; nicotinamide, 0.85; Ca pantothenate, 3.5; pyridoxine·HCl, 0.67; hexahydroxycyclohexane, 180; *p*-aminobenzoic acid, 0.5; folic acid, 0.05; choline chloride, 147; biotin, 0.03; cyanocobalamine, 0.015.
[5] Trivisol, Mead Johnson and Company.

All babies were given 145 Cal. per kg instead of the usual 125 Cal. per kg to allow for the higher caloric requirement when amino acid mixtures are fed instead of natural protein. The feeding was sus-

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[1] Supported by grants from the National Dairy Council, the Wyeth Laboratories and the United States Public Health Service.

TABLE 2

Composition of the amino acid mixtures

	Human milk	FAO
	%	%
L-Alanine	2.67	2.67
L-Arginine	4.58	4.58
L-Aspartic acid	8.79	8.79
L-Cystine	2.14	3.26
L-Glutamic acid	17.56	17.56
L-Glycine	2.06	2.06
L-Histidine	1.76	1.76
L-Isoleucine	6.11	6.85
L-Leucine	11.76	7.8
L-Lysine	7.10	6.85
L-Methionine	1.68	3.58
L-Phenylalanine	4.87	4.55
L-Proline	6.11	6.11
L-Serine	5.34	5.34
L-Threonine	4.58	4.55
L-Tyrosine	4.58	4.58
L-Tryptophan	1.68	2.28
L-Valine	6.63	6.85

pended in water and divided into 6 equal portions given at 4-hour intervals. In order to be certain that the infant received the entire feeding, bottles were rinsed twice with water and the rinse water was also fed.

Metabolic periods were of 4 days duration. Duplicate nitrogen determinations were run on each day's urine collection. Nitrogen determinations were performed in triplicate on the 4-day stool collection. The beginning and end of the stool collection were delineated by carmine and charcoal stool markers. Three of the infants were females for whom it was im-

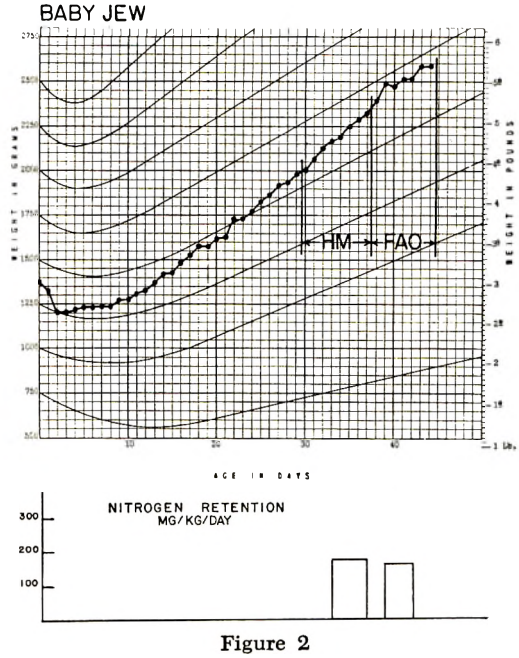
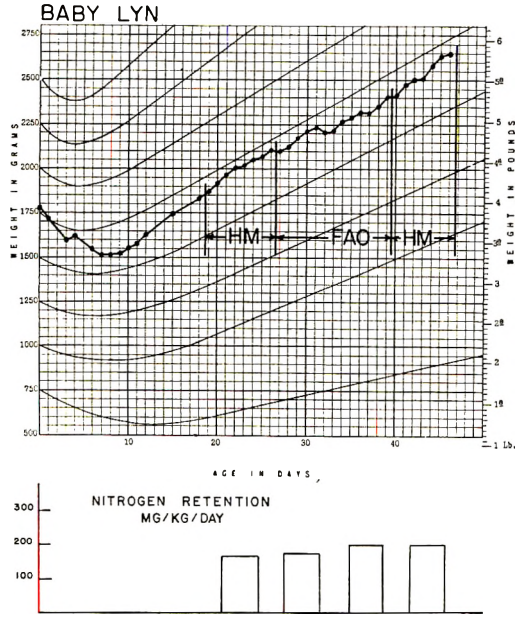


Figure 2



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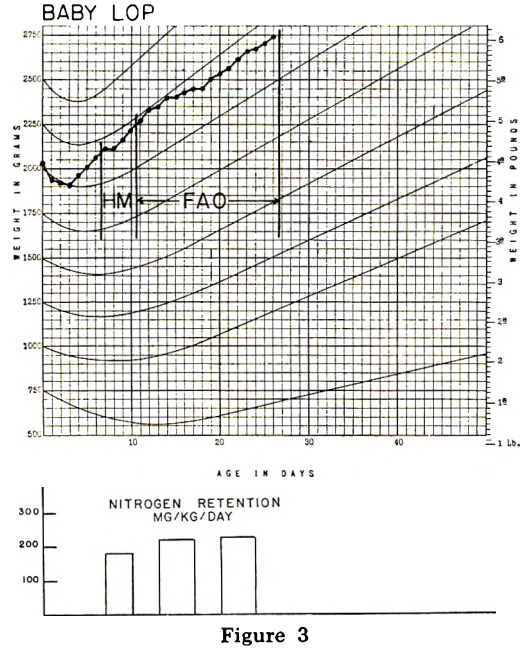

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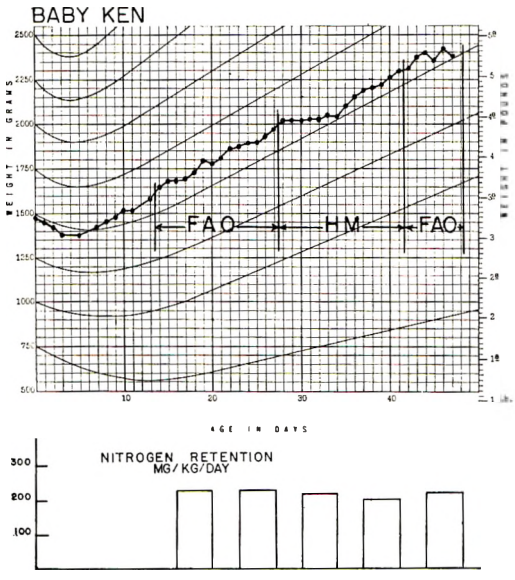

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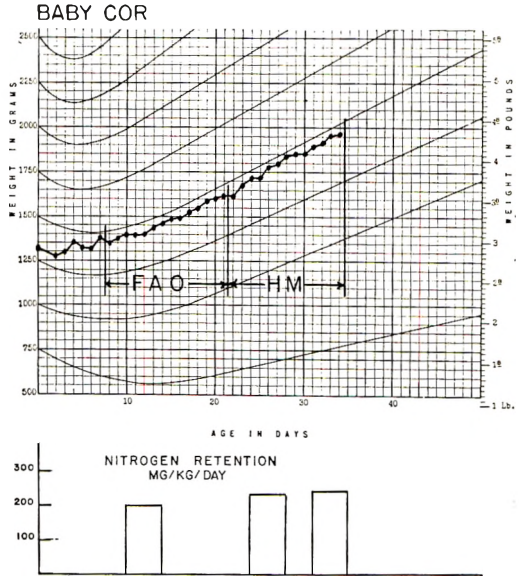

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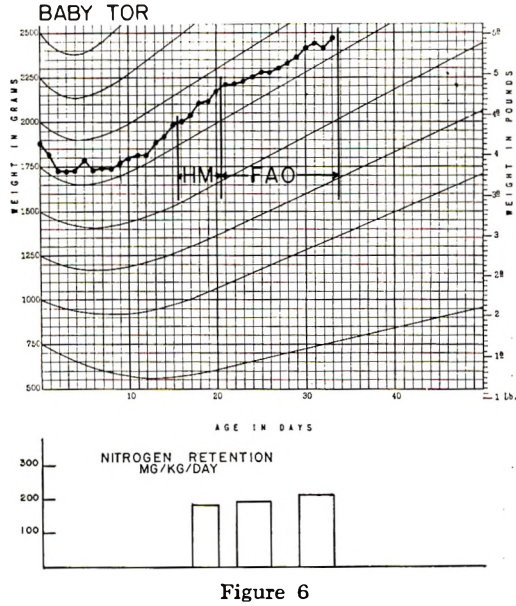

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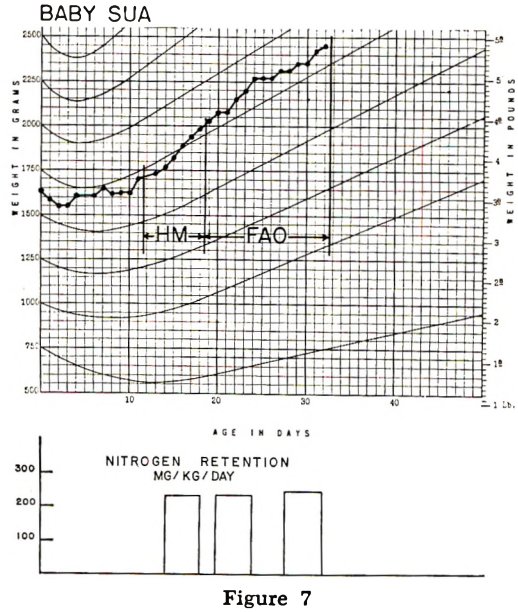

Figure 7

possible to obtain separate urine and stool collections; for these, triplicate determinations were run on the combined stool and urine 4-day collection.

The subjects were 7 premature infants whose birth weights ranged from 1360 to 2030 gm. They were between 6 and 39 days old when they started to receive the experimental diet. All of the subjects remained in good health during the period of study.

Babies were weighed daily and weights plotted on the grid routinely used in our premature unit (Dancis et al., '48). This grid was originally compiled from the weight curves of 100 premature infants

TABLE 3

Metabolic data for 7 premature infants fed two types of diet: one using a human milk (HM) amino acid pattern, and the other the FAO provisional pattern

Subject and date	Average weight	Diet	Nitrogen intake	Urine nitrogen	Stool nitrogen	Nitrogen retention	Biological value[1]	Net protein utilization[1]
	kg		*gm/day*	*gm/day*	*gm/day*	*mg/kg/day*		
Baby LYN								
3/16–19	2.03	HM	0.64	0.21	0.09	167	62	54
3/23–26	2.185	FAO	0.72	0.25	0.08	178	61	54
3/30–4/2	2.345	FAO	0.77	0.24	0.07	196	66	60
4/6–9	2.55	HM	0.82	0.24	0.07	200	68	63
Baby JEW								
2/23–26	2.222	HM	0.70	0.27	0.05	171	58	54
3/2–5	2.495	FAO	0.80	0.33	0.07	160	55	50
Baby ♀ LOP								
3/10–12	2.126	HM	0.69	0.32		174		54
3/16–19	2.4	FAO	0.77	0.24		220		69
3/23–26	2.590	FAO	0.86	0.28		222		67
Baby KEN								
4/27–30	1.725	FAO	0.56	0.12	0.06	220	76	68
5/4–7	1.895	FAO	0.63	0.15	0.05	227	75	69
5/11–14	2.025	HM	0.67	0.15	0.09	212	74	64
5/18–22	2.212	HM	0.72	0.20	0.07	205	70	64
5/25–28	2.38	FAO	0.78	0.20	0.05	222	73	68
Baby COR								
5/25–28	1.410	FAO	0.49	0.16	0.05	198	64	58
6/8–11	1.750	HM	0.57	0.10	0.05	240	80	73
6/15–18	2.0	HM	0.64	0.08	0.06	250	86	79
Baby ♀ TOR								
3/4–7	2.08	HM	0.67	0.29		183		58
3/9–12	2.245	FAO	0.72	0.29		191		60
3/16–19	2.40	FAO	0.78	0.26		217		66
Baby ♀ SUA								
3/23–27	1.858	HM	0.59	0.15		236		74
3/30–4/3	2.180	FAO	0.67	0.16		234		76
4/6–10	2.360	FAO	0.79	0.21		246		73

[1] The figures given represent "uncorrected" biological values and net protein utilization, no correction being made for endogenous excretion on a nitrogen-free diet.

who were fed a formula[2] which provides between 4 and 5 gm of protein per kg per day. It has been our experience that the weight curve of premature infants usually stays in the channel in which birth weight has placed them and deviations from this are of significance.

RESULTS AND DISCUSSION

The protocols of these infants, reproduced in figures 1-7, show the weight curves and the nitrogen retention. Inspection of these graphs does not reveal any significant difference in the performance of these infants between the two types of feedings. All the metabolic data are contained in table 3.

Averages for the uncorrected biologic value and the net protein utilization were similar for the two feedings. The average biologic value was 67 for the FAO pattern and 71 for the human milk pattern. The net protein utilization average was 64 for both feedings. Similarly, the average value for the nitrogen retention with the two types of feedings was very close—210 mg per kg per day for the FAO and 204 mg per kg per day for the human milk amino acid mixture.

[2] Olac, Mead Johnson and Company.

These findings suggest that the FAO pattern of amino acids is nutritionally equivalent to a natural food of high protein quality—human milk—under rigidly controlled conditions.

SUMMARY

Seven premature infants were fed a synthetic diet in which the protein moiety was a mixture of 18 L-amino acids. The amino acids were provided in two ratios—that of human milk and that of the FAO pattern. There were no differences in the weight curves or the amount of nitrogen retained, using the two types of diets. The amino acid pattern of human milk would appear to be quite as satisfactory a standard of reference as the FAO pattern for assaying the nutritional adequacy of protein for the human species.

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Effects of Oleic and Other Fatty Acids on the Growth Rate of Agria Affinis (Fall.) (Diptera: Sarcophagidae)

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In 1956 House and Barlow showed that growth of the parasitoid, *Agria* [=*Pseudosarcophaga*] *affinis* (Fall.), using a chemically defined diet, was accelerated by the addition of lard, and that this effect could be duplicated by a mixture of fatty acids. This raised the question of what fatty acids are needed by *A. affinis*. The present paper reports interrelationships between the fatty acids and their individual effects on the growth of this parasitoid.

MATERIALS AND METHODS

Agria affinis was described by Coppel et al. ('59) and the method of rearing the insects and of testing the various diets, by House and Barlow ('56). Briefly, the larvae were dissected aseptically from the viviparous females, and reared individually and axenically at a constant temperature of 23°C, using chemically defined diets. The composition of the diet (table 1) was changed slightly from that formerly used; other changes for various tests are discussed below.

Fatty acids of high purity were used;[1] the kind, number and amount needed for different diets were dissolved with 250 mg of cholesterol in 1 ml of hot ethanol and emulsified in 100 ml of hot water containing 0.5 ml of polyoxyethylene sorbitan monoleate:[2] 40 ml of emulsion per 100 ml of diet were used. All diets contained the same amount of cholesterol unless otherwise stated, though the fatty acid composition was varied.

About 25 larvae were assigned each diet for each test and at least three replicates of the test were made. The larvae were examined daily and on the day when the percentage of third instar larvae on the entire replicate was nearest 50 (usually the 6th day), the percentage of third instar larvae receiving each diet of the replicate was recorded. The pooled data of the replicates were analyzed statistically.

EXPERIMENTAL

Eight experiments were carried out as follows:

1. *To determine the effects of several fatty acids* substitutions were made for the usual fatty acid mixture shown in table 1. The fatty acid compositions of these diets were as follows:

Group 1
Fatty acid mixture
Mixture minus linolenic acid
Mixture minus stearic acid
Mixture minus linoleic acid
Mixture minus palmitic acid
Mixture minus oleic acid
Oleic acid, 0.192%
Palmitic acid, 0.088%
Stearic acid, 0.04%
Linolenic acid, 0.02%
Linoleic acid, 0.06%
No fatty acid

Group 2
Fatty acid mixture
Oleic acid, 0.192%
Palmitoleic acid, 0.192%
Arachidonic acid, 0.0028%
No fatty acid

The effects of these on growth rate, pupation and adult emergence were determined. The effects on growth are shown in figure 1.

The different fatty acids and mixtures were not equal in their ability to increase growth rate. In both groups comparison

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[1] Obtained from the Hormel Foundation, Austin, Minnesota.

[2] Tween 80, Atlas Powder Company, Ltd. of Canada, Brantford, Ontario.

TABLE 1

Composition of chemically defined diet for Agria affinis

	mg/100 ml distilled water			*mg/100 ml distilled water*	
L-Amino acids (total)		1254.7	Lipids (total)		770
Alanine	64.5		Cholesterol	100.0	
Arginine·HCl	52.9		Linoleic acid	60.0	
Aspartic acid	76.5		Linolenic acid	20.0	
Cysteine·HCl	18.0		Oleic acid	192.0	
Glutamic acid	137.1		Palmitic acid	88.0	
Glycine	17.6		Stearic acid	40.0	
Histidine·HCl	17.6		Polyoxyethylene sorbitan monoleate	270.0	
Hydroxyproline	24.0				
Isoleucine	76.5		Vitamins (total)		11.12009
Leucine	140.3		Biotin	0.00009	
Lysine·HCl	88.2		Ca pantothenate	0.44	
Methionine	47.3		Choline chloride	2.00	
Phenylalanine	70.1		Folic acid	0.34	
Proline	100.2		Inositol	5.96	
Serine	88.2		Nicotinic acid	0.30	
Threonine	47.3		*p*-Aminobenzoic acid	0.9	
Tryptophan	23.6		Pyridoxine·HCl	0.9	
Tyrosine	82.2		Riboflavin	0.16	
Valine	82.6		Thiamine·HCl	0.12	
Inorganic salts (no. 2, USP XII) (total)		66.0	Miscellaneous (total)		1572
Calcium biphosphate $CaH_4(PO_4)_2$	8.96		Glucose	500.0	
Calcium lactate	21.58		Ribonucleic acid	75.0	
Ferric citrate (17.5% Fe)	1.96		Potassium hydroxide 2N	247.0	
Magnesium sulphate	9.04		Agar	750.0	
Potassium phosphate K_2HPO_4	15.83				
Sodium biphosphate	5.76				
Sodium chloride	2.87				

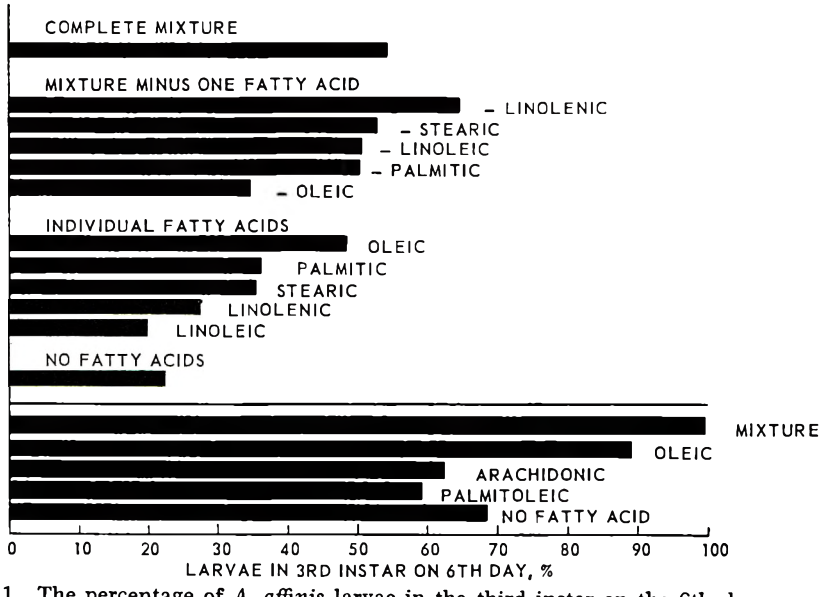

Fig. 1 The percentage of *A. affinis* larvae in the third instar on the 6th day reared with diets of different fatty acid composition.

by statistical analysis (F test) of diets containing oleic acid with diets lacking it showed a high probability ($P < 0.01$) that the faster growth in the presence of this fatty acid was real. Also, within the first group of diets, the faster growth with the diet lacking linolenic acid was probably real ($P < 0.01$). No other comparison showed real differences ($P > 0.05$), nor were significant differences obtained between numbers of pupae and of adults when using these diets ($P > 0.05$).

Though diets containing oleic acid accelerated growth more than those without, other fatty acids, especially palmitic and stearic, caused slight acceleration. Hence it was undecided whether oleic acid alone accelerated growth as much as the mixture of 5 fatty acids. This was investigated as follows:

2. *To compare the effects of oleic acid with those of the mixture,* 6 pairs of diets were tested with fatty acid contents of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2%. One diet of each pair contained oleic acid only, the other the mixture of 5 fatty acids. Only growth rates were measured.

Using the mixture of fatty acids growth was consistently 12 to 15% faster than with oleic acid alone. This difference was statistically significant ($P < 0.01$). Oleic acid cannot replace the complete mixture of fatty acids in the diet. Evidently some other fatty acid is complementary. As shown in figure 1, withdrawal of stearic or palmitic acid from the fat mixture reduced, and addition of either of these increased, the growth rate (though these effects were not as great as those caused by oleic acid). Evidence for linolenic and linoleic acids is conflicting for although linolenic acid stimulated growth when added to the fat-free diet, its removal from the mixture was beneficial, and conversely linoleic acid was not beneficial when added to the fat-free diet but some reduction of growth occurred when it was withdrawn from the mixture. Therefore palmitic and stearic acids are the most probable. The next experiments, therefore, were to determine the relations between oleic acid, palmitic acid and stearic acid.

3. *To determine the effects of palmitic acid with oleic acid,* a factorial design experiment was carried out with 4 levels of palmitic acid and 4 levels of oleic acid. The diets contained zero, 0.044, 0.088 and 0.132% of palmitic acid in all combinations with oleic acid contents of zero, 0.096, 0.192 and 0.384%. As a reference, the diet with 5 fatty acids (table 1) was also tested.

An analysis of variance showed that variation between replicates was significant ($P < 0.01$). Nevertheless, the promotion of growth depended on the levels of both oleic and palmitic acid ($P < 0.01$) and an interaction ($P < 0.01$) occurred between them (i.e., differences between levels of one were not uniform for all levels of the other averaged over all replicates). The fastest growth, equal to that observed with the complete mixture of fatty acids, occurred with about 0.2% of oleic acid and about 0.1% of palmitic acid. The data indicated that lesser amounts of either fatty acids may be needed as the level of the other increases. Thus suitable proportions of oleic and palmitic acids produce growth equal to that when using the diet containing palmitic, stearic, oleic, linoleic and linolenic acids, whereas oleic acid alone was shown to be inadequate in experiment 2.

4. *To determine the effects of stearic acid with oleic acid,* a factorial design experiment was done with 4 levels of stearic acid: zero, 0.04, 0.08 and 0.12%, in all combinations with 4 levels of oleic acid: zero, 0.096, 0.192 and 0.288%. The diet shown in table 1 was also tested as a reference. The effects of these diets on growth were determined.

An analysis of variance showed that variation between replicates was significant ($P < 0.01$). The evidence that stearic acid promoted growth was statistically significant ($P < 0.05 > 0.01$), as was the evidence of interaction ($P < 0.01$).

5 and 6. *To determine the effects of stearic acid with palmitic acid in the presence and absence of oleic acid,* two factorial design experiments were carried out with 4 levels of stearic acid: zero, 0.04, 0.08 and 0.12%, in all combinations with 4 levels of palmitic acid: zero, 0.044, 0.088 and 0.132%. In one of these experiments all diets contained an additional 0.192% of oleic acid. A diet containing the com-

plete mixture of fatty acids was included in each experiment.

An analysis of variance showed that there was a statistically significant interaction ($P < 0.01$) between stearic and palmitic acids in the absence of oleic acid. In its presence there was no such evidence; the interaction was probably masked by the effectiveness of the oleic acid. In the experiment without oleic acid there was an unexplained anomaly in that some of the diets gave growth rates equal to that when using the complete mixture. In the experiment with oleic acid the only effect great enough to have statistical significance was the faster growth with all diets containing palmitic or stearic acids or a mixture of these as compared with the diet containing oleic acid alone. This supports the findings of the previous experiments.

As oleic acid has been reported to partly replace the biotin requirements of the moth *Corcyra cephalonica* St. (Siva Sankar and Sarma, '51) and the mosquito *Aedes aegypti* L. (Trager, '48), and as cholesterol is associated with the absorption of fats in animals (Cook and Thompson, '51) and with oleic acid as a substitute for serum in the culture of certain entozoic amoebae (Griffin and McCarten, '49), the next experiments were designed to show interaction between oleic acid and biotin or cholesterol.

7. *To detect interaction between oleic acid and biotin,* a factorial design experiment was carried out with 4 levels of oleic acid and 4 levels of biotin. Diets were prepared with oleic acid content of zero, 0.15, 0.30 and 0.45% in all combinations with biotin content of zero, 2.2×10^{-8}, 2.2×10^{-7} and 2.2×10^{-6}%. The results are shown in figure 2.

Both oleic acid and biotin, up to a certain concentration, had a growth-promoting effect. The maximum effect of oleic acid occurred at a dietary level of about 0.3%; the maximum effect of biotin occurred at about 1.3×10^{-6}%. Differences in the rates of growth caused by varying content of each are statistically significant ($P < 0.01$), but analysis of variance showed no interaction at the levels of oleic acid and biotin tested.

8. *To detect interaction between oleic acid and cholesterol,* a similar experiment was carried out with diets containing zero, 0.0384, 0.096 and 0.192% of oleic acid in all combinations with cholesterol content of 0.001, 0.005, 0.01 and 0.025%. The results are shown in figure 3.

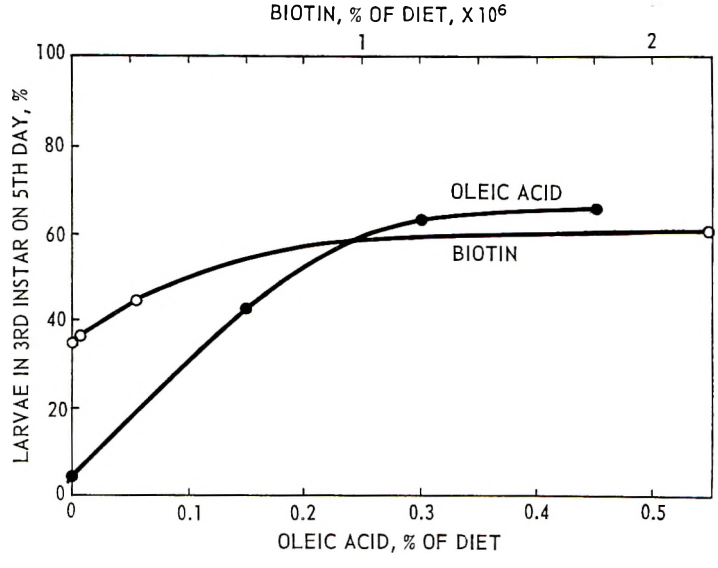

Fig. 2 The percentages of larvae that attained the third instar in 5 days, receiving various levels of oleic acid and biotin in the diet.

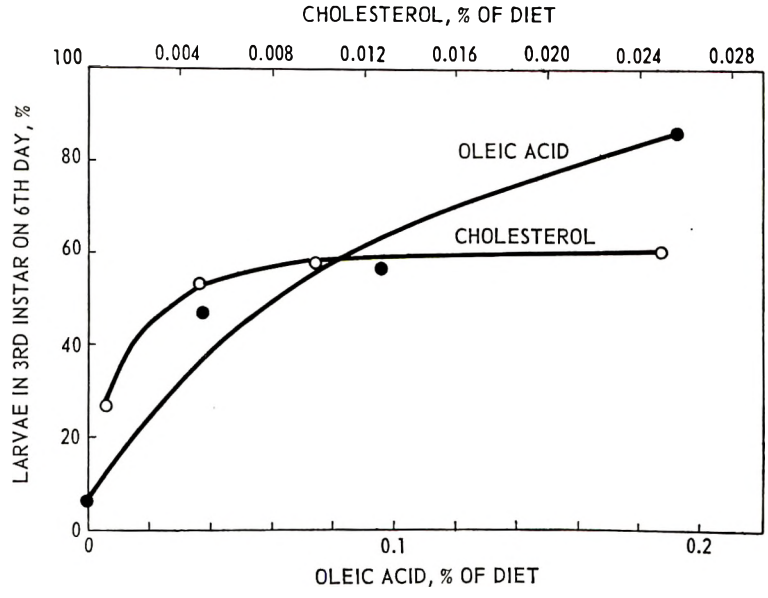

Fig. 3 The percentages of larvae that attained the third instar in 6 days, receiving various levels of oleic acid and cholesterol in the diet.

Both oleic acid and cholesterol promoted growth. The maximum effect of varying levels of oleic acid was not reached in this experiment, but that of cholesterol was reached between the 0.005 and 0.01% levels: greater amounts caused no harm. Analysis of the results showed that differences in the rates of growth caused by varying levels of oleic acid and of cholesterol were statistically significant ($P < 0.01$), but interaction was improbable.

DISCUSSION

Growth of the parasitoid *A. affinis* is accelerated by certain fatty acids, principally oleic acid. There are a few previous reports that fatty acids are essential or have nutritional importance in insects. Linoleic, linolenic and arachidonic acids, but not oleic acid, promoted growth of *Ephestia kuehniella* Zell. (Fraenkel and Blewett, '46, '47) though the principal effects were on the development of wing scales and adult emergence (Fraenkel and Blewett, '46). Linoleic and linolenic acids also promoted moth emergence of *Pectinophora gossypiella* (Saund.); oleic acid was inactive (Vanderzant et al., '57). The European corn borer, *Ostrinia* [=*Pyrausta*] *nubilalis* (Hbn.) apparently needs substances in corn oil: cholesterol, linoleic acid, and α-tocopherol may replace corn oil in its diet (Beck et al., '49). Linoleic acid may be needed by *Loxostege sticticalis* (L.) (Pepper and Hastings, '43). Unsaturated fats possibly have growth-promoting effects on *Calliphora vicina* R.-D. [= *C. erythrocephala* (Meig.)] (Sedee, '56). On the other hand, linoleic and linolenic acids were nutritionally inactive in *Aedes aegypti* (L.) (Golberg and DeMeillon, '48). *Tenebrio molitor* L. synthesizes sufficient linoleic acid for its needs (Fraenkel and Blewett, '47). No evidence was found in the present work that *A. affinis* needed dietary sources of linoleic, linolenic or arachidonic acids.

The maximum growth rate of *A. affinis* when using a mixture of palmitic, stearic, oleic, linoleic and linolenic acids may be superior to that with any single fatty acid, but it is equaled by various mixtures of oleic acid with palmitic or stearic acid. There is good evidence that interactions occur between oleic and palmitic, oleic and stearic, and palmitic and stearic acid. In each case, the data indicate that the need for one may increase as the supply of the other is decreased. Though such interactions are statistically significant (P

< 0.01), variation in these data was such that a wide range of proportional relationships between pairs of fatty acids resulted in equivalent growth rates. Suitable mixtures generally contained about 0.2% of oleic acid, 0.1% of palmitic acid and 0.04% of stearic acid.

Previous work (House and Barlow, '56) showed that the need for fats was independent of a need for carbohydrate. The present work shows that the need for oleic acid is independent of any involvement with biotin or cholesterol at the levels tested. Thus fatty acids may play a specific role in the nutrition of *A. affinis* apart from their calorific value.

SUMMARY

This investigation was prompted by the discovery that lard or a mixture of 5 fatty acids—palmitic, stearic, oleic, linoleic, and linolenic—promoted growth of *Agria affinis* (Fall.), using chemically defined diets. Feeding tests with these fatty acids plus palmitoleic and arachidonic acids showed that, individually, oleic acid had the greatest effect. This was independent of any effects of biotin or cholesterol. Palmitic and stearic acid also increased the growth rate to a lesser extent. Interactions were found between oleic and palmitic, oleic and stearic, and palmitic and stearic acids. The data were inadequate to determine precise requirements, but if the diet contained an optimum amount of oleic acid (0.2%), the addition of between 0.044 and 0.132% of palmitic acid, or between 0.04 and 0.12% of stearic acid, or any combinations of the two in these ranges, resulted in growth equal to that when using the complete mixture. Linoleic, linolenic, palmitoleic and arachidonic acid apparently are not required.

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Multiple Amino Acid Supplementation of White Corn Meal

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After many years of research on the supplementation of proteins with single essential amino acids, the principle evolved that the first limiting amino acid should be added in such an amount that the total is in nutritional balance with the amount of the second limiting amino acid present in the protein. This concept has only recently been applied to the supplementation of a protein with its first two limiting amino acids. Rice, corn meal, and white bread were thus studied. The results of the investigation on the supplementation of rice with lysine and threonine have been published earlier (Rosenberg et al., '59). The study carried out with corn is described here. Using a special factorial design, the amounts of lysine and tryptophan for optimum gain have been determined with rats in a single experiment. In addition, experiments have been carried out to identify the sequence of the next limiting amino acids in corn.

EXPERIMENTAL PROCEDURE

This investigation of the supplementation of corn meal with graded amounts of lysine and tryptophan was designed to establish in a single rat experiment that combination of the two amino acids which elicits maximal attainable weight gain and maximal efficiency of food utilization. In order to achieve this goal, an incomplete factorial design was chosen. The two factors studied were the log ratio and the log product of the total lysine and total tryptophan content of the diets. The principle of the plan is illustrated in figure 1. The total of each amino acid in the diet, in logarithmic scale, is plotted on the axes. The origin of the graph represents the analytically determined amount of the two amino acids in the basal diet. In this log-log plot all points on a line with a 45° positive slope have the same ratio of lysine to tryptophan. All points on a line with a 45° negative slope have a fixed product of lysine times tryptophan. The lysine-tryptophan combinations chosen for experimental study form a grid spaced in equal distances of 0.117 on the log ratio and log product axes. The range of the ratios of lysine to tryptophan is 4:1 to 9:1, which includes the area of the expected maximum. The 18 combinations of lysine and tryptophan used are shown in table 1. A second experiment of the same design was carried out to study the reproducibilty of the results.

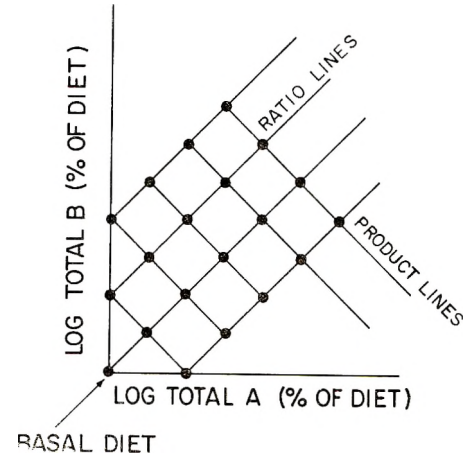

Fig. 1 Design of a feeding experiment with two amino acids aided in varying amounts.

The composition of the basal diet is shown in table 2. The white table corn meal was obtained from a mill in the southern part of the country and contained 1.08% of nitrogen and 11.24% of moisture. The meal was analyzed for the essential amino acids by ion exchange chroma-

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TABLE 1

Effect of supplementation of white corn meal diet with lysine and tryptophan

L-Lysine · HCl	L-Tryptophan	Experiment 1		Experiment 2	
		Average weight gain	Feed/gain	Average weight gain	Feed/gain
%	%	gm		gm	
0	0	14	19.51	4	56.67
0.050	0	28	10.86	15	17.23
0.116	0	14	17.40	10	23.63
0.202	0	2	—	0.5	—
0.023	0.0058	20	14.18	24	13.25
0.080	0.0058	21	13.30	26	12.44
0.155	0.0058	27	10.16	18	14.48
0.254	0.0058	32	9.96	20	12.93
0.050	0.0125	36	9.34	31	10.52
0.116	0.0125	49	7.47	40	8.24
0.202	0.0125	46	7.01	27	10.60
0.315	0.0125	43	7.48	26	10.65
0.080	0.0201	41	8.30	38	9.09
0.155	0.0201	58	6.70	40	8.33
0.254	0.0201	33	9.30	43	7.68
0.116	0.0289	39	8.64	45	7.79
0.202	0.0289	42	8.34	55	6.65
0.155	0.0388	36	8.97	34	9.58

TABLE 2

Composition of basal diet

	% of diet
White corn meal	89.995
Crude soybean oil	4.000
Cod liver oil	1.000
Salt mixture[1]	3.000
Vitamin mixture[2]	2.000
Nicotinic acid	0.005
Total	100.000

[1] For composition of the vitamin and salt mixture see Rosenberg and Culik ('57).

tography procedure (Moore-Stein, '54). Tryptophan was determined microbiologically. The following values were obtained: threonine, 0.23; valine, 0.34; methionine, 0.17; isoleucine, 0.25; leucine, 0.82; tyrosine, 0.25; phenylalanine, 0.31; lysine, 0.18; histidine, 0.23; arginine, 0.29; tryptophan, 0.046%. These data agree well with those published by Edwards and Allen ('58).

Weanling male rats about 21 days old from our colony were used. They were kept during the 5-week experimental period in individual cages with raised screen bottoms. Food and water were supplied ad libitum and weekly records were kept of weight gains and amounts of food consumed. Five animals were assigned to each treatment.

RESULTS

The average growth responses of the groups of growing rats fed the variously supplemented corn meal diets are recorded in table 1. For interpretation of the experimental data the following mathematical model (1) was used:

$$\text{Log weight} = b_0 + b_3 \log W_0 + b_1 \log LT + b_2 \log \frac{L}{T} + b_{11} (\log LT)^2 + b_{22} \left(\log \frac{L}{T} \right)^2 + b_{12} (\log LT) \left(\log \frac{L}{T} \right) \quad (1)$$

in which the b's are constants to be calculated from the responses of each individual animal (but only the averages for each treatment are shown in table 1). L is the total percentage of lysine and T the total percentage of tryptophan in the diet. This model for predicted weight is a more general formula than the model developed for the interpretation of the data on the amino acid supplementation of rice (formula 4 in Rosenberg et al., '59). In the latter study all animals gained considerable weight during the 5-week experimental period. Therefore, the model used predicted weight gains. Feeding diets based on low-protein corn, on the other hand, resulted in moderate gains only and occasionally in small losses of weight for an individual rat. A change in the model was required since the

log of a negative gain or of weight loss cannot be taken. As an additional consideration, it is best to have the response in such a form that its variability is constant for all values of the response. Log of the total weight of the animals met this criterion. The weanling rats used in this study varied somewhat in size. A term for the initial weight W_o, has been added, therefore, to the mathematical model. While the model thus used predicts total weights it should be kept in mind that the prediction is of a relative nature: it is not the absolute weight which is being predicted but the relative change caused by variation of the levels of supplementary amino acids. The absolute weights are, of course, dependent upon many other physiological factors including age and strain of the animals and their response to the basal diet.

The two experiments recorded in table 1 with about 100 rats each were evaluated with this model (1). Regressions of weight and feed consumed were determined for each experiment separately and for the combination. The effects of lysine and tryptophan supplementation on the final weight of the animals and on the feed consumption did not differ significantly ($P = 0.95$) in the two experiments. Therefore, only the results obtained by analyzing the combined data are discussed. Regression analysis using equation (1) gave a good fit of the data as seen in table 3. The evaluated equation is:

$$\text{Log weight} = 0.9837 + 0.5667 \log W_o + 0.0374 \log LT - 0.0274 \log \frac{L}{T} - 0.0135 (\log LT)^2 - 0.0222 \left(\log \frac{L}{T} \right)^2 + 0.0230 (\log LT) \left(\log \frac{L}{T} \right). \quad (2)$$

Within the limits of the data the contour map of this equation is shown in figure 2. For this graph the initial weight was fixed at 60 gm and the contours labeled with predicted mean weight gain. On the basal corn meal diet, a gain of only 9 gm is predicted. Adding about 0.2% of L-lysine and 0.02% of L-tryptophan results in an average gain of 44 gm. This calculated maximal weight gain results when the corn diet has a total of 0.35% of L-lysine and 0.063% of L-tryptophan. The ratio of lysine to tryptophan at the greatest gain is 5.5:1. This value is considered in excellent agreement with Rose's ratio of 5:1 ('37). In this special combination the two amino acids are properly balanced in the diet used. *Proper balance* has been defined (Rosenberg et al., '59) as that combination of amino acids which gives maximal performance for any given product. In this experiment, proper balance at

TABLE 3

Regression analysis tables for rats fed a white corn meal diet supplemented with lysine and threonine

Source of variation	Degrees of freedom	Sum of squares		Mean square	F ratio	Critical values	
		Around the mean	%			F 0.99	F 0.95
			Weight gain				
Total	157	1.54740	100.00				
Regression[1]	6	0.56728	36.66	0.09454	18.36	3.43	
	1	0.05323	3.44	0.05323	10.33	7.51	4.15
Residual	150	0.92689	59.90	0.00618			
Lack of fit	120	0.77231		0.00643	1.25		1.66
Error (replicates)	30	0.15458		0.00515			
			Feed/consumed				
Total	157	1.32335	100.00				
Regression[1]	6	0.28386	21.45	0.04731	9.39	3.43	
	1	0.04301	3.25	0.04301	8.53	7.51	4.15
Residual	150	0.99648	75.30	0.00664			
Lack of fit	120	0.84529		0.00704	1.40		1.66
Error (replicates)	30	0.15119		0.00504			

[1] The 6 d.f. are for the ratio equation and the additional one degree of freedom completes the full-quadratic equation. (For definition of terms, see Rosenberg et al., '59).

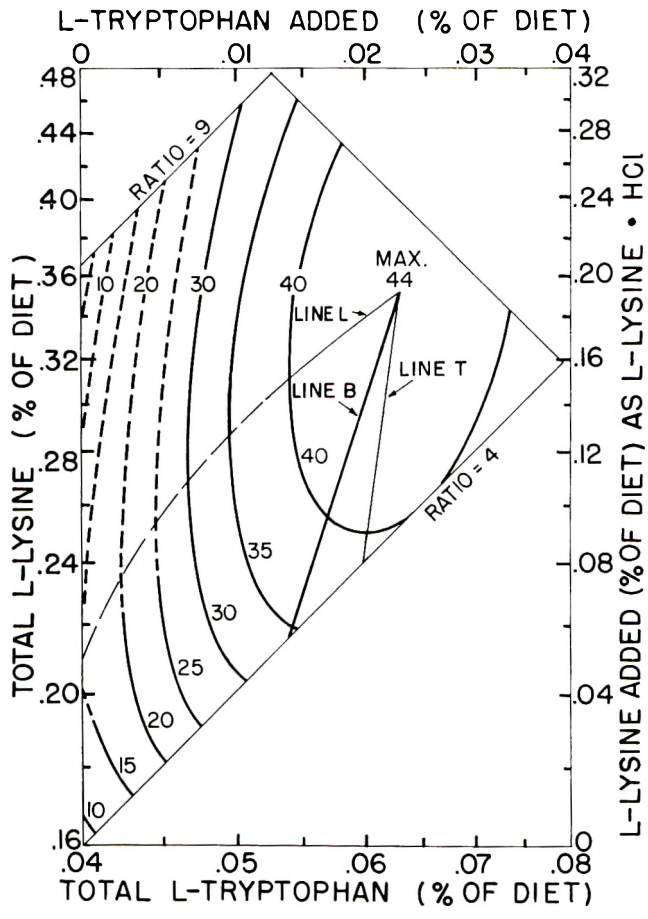

Fig. 2 Weight gain (grams) contours for 60-gm male weanling rats fed for 5 weeks corn meal diets supplemented with lysine and tryptophan. Based on equation 2.

suboptimal levels of supplementation occurs at ratios somewhat lower than 5.5:1. This effect is well established from these data. Line B shows proper balance is at a ratio of about 4:1 with lysine addition of 0.06%.

When the corn meal diet was supplemented only with graded amounts of lysine the best gain, a 50% improvement over the basal diet, was realized with 0.05% added. Supplementation with larger amounts of lysine causes an imbalance (Harper, '58) and results in lower gains. The contours are dotted in this region in order to show that these data were not included in the analysis. This treatment is necessary since the imblance is the result of a new force not included in the mathematical model. Line L shows diets which result in the largest gains when the tryptophan is held constant at any amount and the amount of lysine varied. Line T shows the amount of tryptophan for maximal gain with fixed lysine level.

An equation similar to (1) predicts efficiency of food utilization. Calculations showed it to be so close to weight gains in pattern that no plot is presented. The efficiency ranges from 3.3% with the basal diet to 12.6% at the maximum.

The various phenomena found were similar to those observed in the investigations with rice, particularly the occurrence of separate lines for proper balance and for optimal responses to single amino acid additions. Similar findings were made with bread diets as previously pointed out;

together this supplies convincing evidence that the procedures used are correct.

The rate of growth observed when the corn meal diet was properly supplemented with lysine and tryptophan was considerably below that obtained with the stock diet. In spite of the low protein content of the corn meal diet, better growth should be realized if all amino acid deficiencies could be eliminated. It was of interest, therefore, to determine to what extent supplementation with the next limiting amino acids would improve growth and food utilization. Since, however, the third and subsequent limiting essential amino acids in corn have not been identified, the experiment shown in table 4 was carried out. On the basis of the present knowledge of the amino acid requirement of the growing rat and of our analysis of the amino acids of corn meal, threonine and isoleucine, and possibly also methionine and valine were candidates. In order to obtain largest possible growth response to the next limiting amino acids, the total lysine and tryptophan in the basal diet were raised to 0.48 and 0.1%, respectively, by the addition of 0.40% of L-lysine hydrochloride and 0.06% of L-tryptophan.

The factorial design used in this experiment and the results are shown in table 4. Analysis of variance indicates that all these amino acids are involved in significant interactions. Therefore interpretation is made directly from the data. Neither one of the 4 amino acids added singly at the 0.05% level improved the diet. In fact, each addition created a greater imbalance than existed previously. When two of these 4 amino acids were added at the same level, the combination of threonine and isoleucine gave a considerable improvement in growth and efficiency of food utilization. All the other possible combinations did not permit the animals to grow as well as without these additions. In the absence of more extensive and quantitative data, it may be assumed that there is no single amino acid which is in third place in the sequence of essential amino acid deficiencies in the white corn meal protein used in these experiments. After lysine and tryptophan, the two amino acids threonine and isoleucine appear to be about equally deficient. In the presence of these 4 amino acids, methionine gave a growth response, and valine seemed to provide better growth when methionine was present. These effects were not large enough to be significant but suggest that methionine and valine should be further evaluated. Phenylalanine and histidine should also be included in future studies.

Table 5 records the results of an experiment on the effect of the addition of graded levels of isoleucine to the unsup-

TABLE 4

Effect of 0.05% amino acid additions to white corn meal diets supplemented with 0.40% of L-lysine·HCl and 0.06% of L-tryptophan

Group	Supplementation	Av. weight gain	Feed/gain
		gm	
1	None	61	6.17
2	Threonine	43	7.38
3	Isoleucine	45	6.86
4	Valine	46	6.95
5	Methionine	46	6.91
6	Threonine + isoleucine	105	4.36
7	Threonine + valine	35	8.03
8	Threonine + methionine	45	6.58
9	Isoleucine + valine	54	6.14
10	Isoleucine + methionine	53	6.05
11	Valine + methionine	54	6.16
12	Threonine, isoleucine + valine	111	4.47
13	Threonine, isoleucine + methionine	120	4.13
14	Isoleucine, valine + methionine	57	6.15
15	Threonine, valine + methionine	70	5.20
16	Threonine, isoleucine, valine + methionine	130	4.13
17	Unsupplemented corn meal diet	21	14.59

TABLE 5

Effect of isoleucine addition to various corn meal diets

Group	Supplementation				Av. weight gain	Feed/gain
	L-Lysine · HCl	L-Isoleucine	L-Tryptophan	L-Threonine		
	%	%	%	%	*gm*	
1	—	—	—	—	18.6	15.8
2	—	0.0125	—	—	19.2	14.5
3	—	0.025	—	—	4.4	50.9
4	—	0.05	—	—	13.0	20.1
5	—	0.10	—	—	10.6	23.2
6	0.025	—	—	—	23.4	12.0
7	0.05	—	—	—	28.4	10.4
8	0.10	—	—	—	20.6	11.8
9	0.10	0.0125	—	—	6.2	35.5
10	0.10	0.025	—	—	10.0	22.7
11	0.10	0.05	—	—	9.2	24.2
12	0.10	0.10	—	—	16.0	15.3
13	0.40	0.0125	0.06	—	43.8	7.2
14	0.40	0.025	0.06	—	38.2	7.9
15	0.40	0.050	0.06	—	40.6	7.4
16	0.40	0.100	0.06	—	43.2	7.1
17	0.40	—	0.06	0.05	41.2	6.9
18	0.40	0.050	0.06	0.05	91.0	4.8

TABLE 6

Effect of threonine and typtophan on lysine-supplemented corn meal diets

Group	Supplement	Av. weight gain	Feed/gain
		gm	
1	None	11.3	27.7
2	0.125% L-lysine·HCl	12.5	18.8
3	0.125% L-lysine·HCl + 0.02% DL-threonine	7.5	39.6
4	0.125% L-lysine·HCl + 0.015% L-tryptophan	44.3	8.2

plemented white corn meal diet and to the diets supplemented with lysine, and with the combination of lysine and tryptophan. No beneficial effect was seen in any of these treatments. However, the combination of lysine and tryptophan with isoleucine and threonine again performed much better than any of the other treatments. Finally a high-spot experiment, shown in table 6, was carried out to confirm that tryptophan and not threonine is the second limiting amino acid in corn meal protein.

DISCUSSION

Amino acid supplementation of corn has been investigated many times since the initial research by Osborne and Mendel ('14) and Hogan ('17) which showed that lysine as well as tryptophan was needed to obtain a growth response. Mitchell and Smuts ('32) obtained slightly improved growth with lysine whereas tryptophan addition showed no improvement until after the addition of lysine. When the supplementation of corn meal with the first limiting essential amino acid was reinvestigated recently, lysine produced beneficial results when added up to a level of 0.05% of the diet (Rosenberg, '59). Corn meals from different sources and having different protein content have been tested in similar fashion including yellow whole and degerminated corn and have been found to require similar small amounts of lysine. White corn meal of low protein content was used in the present study. Maximal response from lysine supplementation alone was obtained again at the 0.05% level.

The supplementation of corn with amino acids in addition to lysine and tryptophan has also been studied in several laborator-

ies. Waddell ('58), who reviewed this work, said, "It seems fair to state that no biological tests of sufficient accuracy have been done to identify definitely the third limiting amino acid of corn protein." The present study indicates that there is probably no single amino acid which is the third limiting, but that the third rank is taken by the combination of threonine and isoleucine. Sauberlich et al. ('53), in their studies on amino acid supplementation of low-protein corn diets to which they had added 0.3% of cystine, obtained maximal growth responses when threonine, isoleucine and valine were added in addition to lysine and tryptophan. They did not attempt to identify the third or 4th limiting amino acid but intended to build up the amino acid content of corn to the requirement as stated by Rose ('37).

Benton et al. ('55) have confirmed the finding of Sauberlich and co-workers ('53) that an isoleucine deficiency limits the growth of rats fed corn diets supplemented with lysine, tryptophan, valine and threonine. Harper et al. ('55) observed an excess of dietary leucine to retard the growth of rats fed low-protein diets or diets deficient in isoleucine. The addition of isoleucine to such diets overcame, to a large extent, the growth-retarding effect of an excess of L-leucine. They suggested, then, that leucine can act as an antimetabolite of isoleucine in the rat and can thereby increase the requirement of the rat for isoleucine. Under the conditions of the present experiments supplementary isoleucine did not improve the basal corn meal diet, nor this diet supplemented with lysine alone or with the combination of lysine and tryptophan. On the other hand, a growth response was obtained when isoleucine together with threonine was added to the lysine- and tryptophan-supplemented corn meal diet. It would seem then that isoleucine and threonine are about equally limiting in the protein of the white corn meal used. As stated earlier, this observation might well be interpreted to show that these two amino acids, isoleucine and threonine, together take the third place among the limiting amino acids. This result is not unexpected on the basis of our analytical determination of the essential amino acids in the white corn meal, but had not been expected due to the limited availability of the amino acids from corn meal.

Thus it may not be necessary to use the postulate of a leucine-isoleucine antimetabolite action in order to explain the results of these experiments. The interpretation of the isoleucine response as the result of an antagonism with leucine would require not only that the level of leucine be high enough to cause interference but also the additional hypothesis that the level of isoleucine be low enough so that this antagonism would make isoleucine appear to be the most limiting amino acid in the diet only after lysine, tryptophan and threonine have been added. The improbability of this hypothesis would seem to eliminate the interpretation of an antimetabolite activity. It should be pointed out, also, that the addition of threonine alone and of isoleucine alone to the lysine- and tryptophan-supplemented diet resulted in a growth depression which was overcome when the combination of threonine with isoleucine was fed. Thus, an antagonism between threonine and isoleucine could also be considered. We prefer, however, to look upon these phenomena as expressions of amino acid imbalances. If there was a true metabolic antagonism between isoleucine and leucine in the corn meal, it would seem that isoleucine supplementation should be beneficial whenever corn or corn protein is fed. This was not the case, however, in the present experiments. Further work should be carried out to study these and other amino acid interrelationships.

SUMMARY

A white corn meal diet was supplemented with graded levels of lysine and tryptophan in an experiment of incomplete factorial design based on the ratio and the product of the two first limiting amino acids. From the growth and efficiency of food utilization of weanling rats receiving the 18 different combinations used, the amounts of lysine and tryptophan required for optimal results were calculated. Maximal performance was predicted to occur at a ratio of 5.5 to 1 for lysine to tryptophan. This result confirmed the 5:1 ratio suggested by Rose.

Methionine, threonine, isoleucine and valine were evaluated as the third limiting essential amino acid in corn. The addition of none of these singly improved the growth of the weanling rat fed the corn meal diet, appropriately supplemented with lysine and tryptophan. Among the combinations of two of these amino acids only threonine and isoleucine caused an appreciable response, suggesting that these two amino acids are about equally limiting in corn. Under the conditions of these experiments no evidence for a leucine-isoleucine antagonism was observed.

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Supplementation of Bread Protein with Lysine and Threonine

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The concept of the *ideal protein* provides that the amounts of all essential amino acids are balanced against each other and against the nonessential amino acids according to the nutritional needs of the organism. Although there are some differences between species, the growing rat is generally considered to be well suited for the study of amino acid requirements and interrelationships in nonruminant mammalian nutrition (Osborne et al., '19; Hegsted and Worcester, '47; Howe et al., '60). Accordingly, this animal species has been used widely for the exploration of the amino acid deficiencies in various proteins and for studies to overcome these deficiencies by supplementation with the limiting amino acids. Depending upon the quantity of supplementary amino acid used, the existing imbalance may be reduced, corrected or supplanted by a new imbalance. The principle of balancing the amino acid in shortest supply, the first limiting amino acid, against the next limiting amino acid has only recently been applied to studies of multiple amino acid supplementation. The first two limiting essential amino acids in rice, in white corn meal and in bread were thus studied. In the investigation on rice (Rosenberg et al., '59), a procedure was developed to predict by calculation the amount of each of the two amino acids needed for optimal growth and efficiency of food utilization as well as their proper ratio to each other at any level of supplementation. In the corn meal report (Rosenberg et al., '60), a simple experimental design was shown to yield in a single experiment essentially all the desired information. The bread study, described here, involved the application of the same design. The data from these experiments were evaluated by means of the methods developed with rice and corn meal. The results are in accord with the findings of the earlier investigations. The methods used should, therefore, be applicable to the multiple amino acid supplementation of all proteins.

EXPERIMENTAL PROCEDURE

The composition of the basal diet is shown in table 1. The source of protein was a commercial white bread containing 3% of nonfat dry milk. This diet contained 1.5% of nitrogen, 0.21% of lysine and 0.25% of threonine. The various combinations of lysine and threonine used are shown in table 2. The lysine-threonine combinations chosen for the study form a square grid on logarithmic coordinates; the side of each square is 0.114 in log units. This is identical with taking the lowest ratio of 1:1 and multiplying by 1.3 to obtain the second ratio, and so on. Product lines for the grid are established in this same way. All calculations in planning such an experiment are more easily performed by adding logs. In the first

TABLE 1
Composition of basal diet

	% of diet
White bread (air-dried, ground)[1]	63.5
Hydrogenated vegetable fat	9.5
Cod liver oil	0.5
Potato starch	16.5
Glucose	5.0
Salt mixture[2]	3.0
Vitamin mixture[2]	2.0
	100.0

[1] A commercial white bread containing 3% of nonfat dry milk.
[2] The composition of the vitamin and salt mixtures is described by Rosenberg and Culik ('57).

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TABLE 2

Effect of supplementation of 63.5% dry bread diet with lysine and threonine

Supplementation		Ratio of lysine to threonine	Experiment 1		Experiment 2	
L-Lysine	L-Threonine		Av. weight gain	Feed/ gain	Av. weight gain	Feed/ gain
%	%		*gm*		*gm*	
0	0	0.8	22	14.75	32	11.09
0.050	0	1.0	36	10.11	—	—
0.125	0	1.3	82	6.27	99	5.44
0.223	0	1.7	133	4.56	135	4.31
0.350	0	2.2	—	—	144	4.24
0.085	0.035	1.0	56	7.34	—	—
0.170	0.035	1.3	108	5.10	136	4.67
0.282	0.035	1.7	149	4.38	162	4.08
0.427	0.035	2.2	—	—	193	3.65
0.125	0.075	1.0	80	5.94	—	—
0.223	0.075	1.3	162	4.28	168	4.06
0.350	0.075	1.7	223	3.42	189	3.50
0.515	0.075	2.2	—	—	208	3.35
0.170	0.120	1.0	108	5.18	—	—
0.282	0.120	1.3	150	4.36	177	3.86
0.427	0.120	1.7	212	3.34	204	3.32
0.614	0.120	2.2	—	—	192	3.37
0.223	0.173	1.0	137	4.51	—	—
0.350	0.173	1.3	195	3.58	193	3.40
0.515	0.173	1.7	199	3.47	218	3.25
0.731	0.173	2.2	—	—	191	3.47
0.282	0.232	1.0	141	4.53	—	—
0.427	0.232	1.3	214	3.42	194	3.45
0.614	0.232	1.7	—	—	187	3.48
0.350	0.300	1.0	191	3.59	—	—
0.515	0.300	1.3	—	—	182	3.51
Stock diet			—	—	255	2.63

experiment the range of the ratios of lysine to threonine was from 1:1 to 1.7:1. In the second experiment the lysine:threonine ratio of 2.2:1 was added and the 1:1 dropped. Five weanling male rats were used per treatment in each experiment, with a total of about 200 animals. The animals and their management were the same as described in earlier reports.

RESULTS

The results of the two experiments are shown in table 2. The average growth responses of the groups of growing rats are recorded but the total body weight and total food consumption of each individual animal were included in the interpretation of the results. For this purpose the general formula (1) of the earlier study on white corn meal was used. The results of the first experiment indicated that lysine and threonine might be balanced near or beyond the highest ratio used. Therefore, the levels of lysine and threonine were changed slightly in the second experiment so as to surround the maximum predicted from the first experiment with diets of higher lysine to threonine ratio. In turn the diets with the lowest ratio in the first experiment were dropped. The results of the two experiments were in reasonable agreement. Since analysis of the data from the white corn meal investigation had shown that the effects of the amino acids did not differ significantly between experiments, the combined data of the present study were analyzed. All results within the planned experiment were used. The data from the basal unsupplemented diet, how-

ever, were not included in the analysis since the ratio of lysine to threonine in this diet was lower than the limit of the plan. Regression analysis gave a good fit of the data. The evaluated equation is:

$$\text{Log weight} = 1.6485 + 0.4072 \log W_o + 0.0812 \log LT + 0.1062 \log \frac{L}{T} - 0.0372 (\log LT)^2 - 0.0951 (\log \frac{L}{T})^2 - 0.0172 (\log LT)(\log \frac{L}{T}) \quad (1)$$

Within the limits of the data the contour map of this equation is shown in figure 1. For this graph the weight of the weanling animals at the start of the experiments was fixed at 60 gm and the contours were labeled with predicted mean weight gain (Rosenberg et al., '60). Using the diet with the smallest lysine supplementation and no threonine added, a weight gain of 33 gm is predicted. The basal diet gives even less gain but is outside the analysis as stated earlier. Adding 0.5% of L-lysine and 0.17% of L-threonine results in a mean gain of 214 gm. This calculated maximal weight gain is obtained when the diet contains a total of 0.71% of L-lysine and 0.42% of L-threonine. The ratio of lysine to threonine at the greatest gain is 1.67:1. This is the same ratio as found by Rose ('37). *Proper balance* has been defined (Rosenberg et al., '59) as that combination of amino acids which gives maximal performance for a given product of these acids. In the experiments the *proper balance line*, B, coincides with the 1.67% ratio line. Actually, the data show a small departure of the proper balance line, B, from the ratio line at the smaller levels of amino acid supplementation. This departure, however, is not significant.

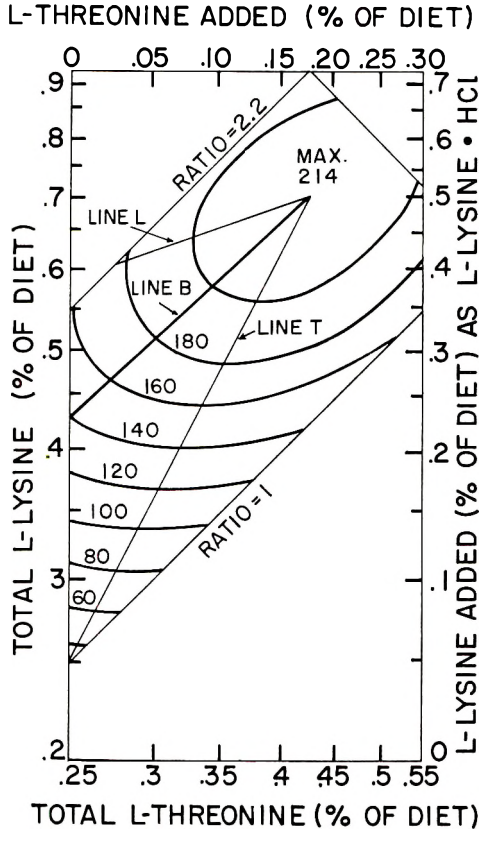

Fig. 1 Weight gain (grams) contours for 60 gm male weanling rats fed for 5 weeks bread (wheat) supplemented with lysine and threonine.

Lines L and T in figure 1 are the *optimal response lines* resulting from varying one amino acid while keeping the other constant. Line L shows the performance of diets which result in the largest gains when threonine is held constant at any level and lysine is increased. For example, when only lysine is added to the basal diet the maximal gain is predicted with 0.58% of total L-lysine. This almost coincides with the diet in which no threonine was added and the total lysine was 0.55%. Line T shows the amount of threonine for maximal performance with fixed lysine level.

Efficiency of food utilization follows the pattern of weight gains. Greatest efficiency is obtained at about the same ratio of the amino acids. The food efficiencies range from 9% using the diet with only the small lysine supplementation to 32% at the maximum.

DISCUSSION

The supplementation of commercial white bread with lysine was studied systematically about 10 years ago (Rosenberg and Rohdenburg, '52). The addition of all the needed nutrients, salts, fat and vitamins, reduced the amount of dried ground bread in the diet to 90%. This diet plus graded levels of lysine was fed to groups of weanling rats. When a total of about 0.7% of lysine was used in the diet, the animals

grew as well as those receiving a stock diet. It was concluded, therefore, that "so far as rat growth is concerned, the only important amino acid deficiency in commercial bread is lysine."

After Pecora and Hundley ('51) had shown that the nutritive value of the protein of rice could be greatly improved by the addition of lysine and threonine, Sure ('52) carried out similar rat growth experiments with whole wheat. Feeding a whole wheat diet at an 8% protein level, Sure observed a substantial increase in growth and protein efficiency when lysine alone was added, but obtained further increases by adding valine and threonine. A calculation of the amounts of valine and threonine present in the bread diet used by Rosenberg and Rohdenburg ('52) showed that it contained only 71 and 75%, respectively, of the amounts considered necessary to support normal growth according to the requirement figures of Rose et al. ('49). Maximal growth equivalent to that obtained with a stock diet occurred when the total lysine of the bread diet was about 75% of the requirement observed by Rose. This suggested that perhaps the minimal requirement figures were lower under the conditions of these experiments or that the growth obtained with the stock diet was suboptimal. In any event, the supplementation of the 90% bread diet with these essential amino acids was investigated (Rosenberg et al., '54). When 0.8% of lysine was added to the basal 0.29% lysine-containing diet, thus providing at least Rose's 1% requirement figure, growth and food efficiency similar to that found with the stock diet was obtained. When either valine or threonine was also added to bring the total up to 90 to 100% of Rose's requirement figures no additional growth was obtained. Neither did the addition of either valine or threonine alone in the absence of supplementary lysine improve the performance of the basal diet. Finally, in the presence of a suboptimal level of lysine no beneficial effect was seen from the addition of valine and threonine. These experiments aided considerably in formulating the general rules of amino acid supplementation. They showed that the nutritional requirement for the second limiting amino acid must have been satisfied since a response from a potential second limiting amino acid was not obtained. Since Sure ('52) had obtained a response from the addition of a second or third essential amino acid, this response could have been possible only because there was less of the second limiting amino acid present than in the diet used by Rosenberg and Rohdenburg ('52). This is precisely the condition that existed. The latter authors had used a bread diet of 12.5% protein content before the addition of supplementary amino acids whereas Sure ('52) had fed a whole wheat diet containing 8% of protein. Although the quality of the protein was slightly different, the prevailing factor is the different level of protein as pointed out by Rosenberg et al. ('54). An extensive study by Hutchinson et al. ('59) with bread protein has also confirmed these conclusions.

In our other studies on the supplementation of protein with its first two limiting essential amino acids, rice and corn meal were used. Because of their inherent low protein content they were ideally suited for such investigations. In the case of bread the protein content of the diet had to be lowered from the previously used 12.5% protein level in order to permit the second limiting amino acid to become a limiting nutrient (for additional discussion on the choice of level of protein, see Rosenberg, '59). The diet used in the present study contained 9.3% of protein ($N \times 6.25$) from 63.5% of dry bread (table 1).

In the rice study different *lines of optimal response* were observed when one amino acid was held constant and the other varied. The same phenomenon was also seen with corn meal. In the present investigation there are again two lines of optimal response, line L on figure 1 when lysine is varied and threonine is held constant and line T when threonine is varied and lysine is constant. With these data on the three different proteins in rice, corn meal and bread, the existence of two separate lines of optimal responses of single amino acids must be considered well established.

Supplementation of the 90% dry bread diet with lysine (Rosenberg and Rohdenburg, '52) revealed that maximal response was obtained with a diet which contained

TABLE 3

Regression analysis table for rats fed 63.5% dry bread diet supplemented with lysine and threonine

Source of variation	Degrees of freedom	Sum of squares: Around the mean	Sum of squares: %	Mean square	F ratio	Critical values: F 0.99	Critical values: F 0.95
			Weight gain				
Total	177	2.44493	100.00				
Regression[1]	6	2.02318	82.75	0.33720	130.70	4.01	
	1	0.00538	0.22	0.00538	2.09		4.41
Residual	170	0.41637	17.03	0.00244			
Lack of fit	152	0.37516		0.00247	0.96		1.95
Error (replicates)	18	0.04121		0.00258			
			Feed/consumed				
Total	177	1.21324	100.00				
Regression[1]	6	0.77878	64.19	0.12980	66.22	4.01	
	1	0.00012	0.01	0.00012	0.06		4.41
Residual	170	0.43434	35.80	0.00255			
Lack of fit	152	0.39908		0.00263	1.34		1.95
Error (replicates)	18	0.03526		0.00196			

[1] The 6 d.f. are for the ratio equation and the additional one degree of freedom completes the full-quadratic equation. (For definition of terms, see Rosenberg et al., '59.)

0.67% of L-lysine. The 90% basal bread diet contained 0.31% of lysine and 0.37% of threonine. As seen from figure 1 the earlier value agrees well with the predicted value on line L for 0.37% of threonine. These observations supply further proof that total protein in a diet is less important than the levels of the limiting amino acids.

In the present study on bread the *line of proper balance* between lysine and threonine coincides with the 1.67 constant ratio line. This is evidence that the analytical values for lysine and threonine in the basal bread diet are essentially correct and the two amino acids are almost completely available. This was not the situation in the precooked rice nor in the white corn meal.

The ratio of 1.67 for lysine to threonine at the optimal level of amino acid supplementation is in agreement with the ratio of Rose's ('37) amino acid requirements for the growing rat but is lower than the 2.00 ratio of Rose et al. ('49). In the rice diet a smaller ratio had been found to give optimal response. The difference between these two ratios is believed to be real as each value is based on well-founded experimental evidence. Although the reason for this difference is not known, the limited availability of one or more of the amino acids in the protein is the probable cause. When discussing the data on the supplementation of rice we suggested: "It is conceivable that there is a specific balance characteristic for each protein, as the absolute amount of any one essential amino acid required for best balance may be affected by the amount of all the other amino acids in the test protein." Further research is needed to clarify this situation.

SUMMARY

With a bread diet of low protein content (1.5% of nitrogen) the responses of growing rats to various levels of lysine and threonine supplementation were studied. An incomplete factorial design based on ratio and product of the two essential amino acids was used for the two experiments reported. From the results, optimal performance was calculated to occur at a ratio of lysine to threonine of 1.67 to 1. This figure is in agreement with the requirement data of Rose but is higher than the 1.4:1 ratio determined recently for rice.

In these studies on the protein of bread as well as in the earlier studies on the proteins of rice and of white corn meal there were observed at suboptimal levels of

amino acid supplementation two different sets of maximal responses, one for each amino acid at fixed levels of the other amino acid. "Proper balance" between the two amino acids, at any level of supplementation, occurred when maximal response was obtained from a given product of the two amino acids.

The present study confirmed that threonine is the next limiting amino acid in bread protein after lysine. However, it was necessary to reduce the level of bread protein in the diet below 12.5% (dry weight) in order to permit threonine to become a limiting nutrient. At a 9.3% protein level, the maximal weight gain obtained by supplementation with lysine alone was about 75% of that reached with the optimal combination of lysine and threonine.

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Fat Utilization in the Fluoride-Fed Rat[1]

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A severe depression in the growth of fluorotic rats fed ad libitum was noted by Miller and Phillips ('55) when the fat content of the diet was raised from 5 to 15%. This effect was unrelated to the chain length of the fat fed and apparently was not attributable to a lack of coenzyme A for fatty acid utilization.

Muhler and co-workers (Bixler and Muhler, '60; Buttner and Muhler, '57, '58) have studied this relationship further, and have advanced the hypothesis that the increased toxicity may be related in part to an increased retention of fluorine in the soft tissues of animals fed a high-fat ration. Sievert and Phillips ('59), in further studies of the fluorine-fat interrelationship, demonstrated that the activity of the kidney-fatty acid oxidizing system was markedly inhibited in fluorotic rats. This change was accompanied by structural damage to the kidney so that the specificity of the effect could not be established.

Their studies indicated that the decreased growth of fluorotic rats fed ad libitum and receiving a high-fat diet was due to a voluntary reduction in food intake. When compared with pair-fed control rats, the fluorotic animals exhibited a decrease in growth rate which was presumably caused by a marked excretion of nitrogen, fat and total dry matter in the feces.

The excretion of fecal fat was most drastically affected, and three possible causes were advanced: an increase of metabolic fat, a decrease of lipolysis in the intestine or a poor absorption of digested lipid material. The objective of the studies to be reported here was to elucidate the causes for this high level of fecal fat excretion.

METHODS

Female albino rats of the Holtzman strain were used for all experiments. These animals were housed in galvanized iron or stainless steel cages with raised screen bottoms, in an air conditioned animal room. Food consumption was measured and fecal collections made quantitatively when needed.

The animals were fed either a stock diet composed of natural products or various semipurified diets (table 1). The vitamin mix used contained per kilogram of diet: choline chloride, 1 gm; and (in milligrams) inositol, 100; Ca pantothenate, 20; niacin, 10; menadione 4; riboflavin, 3; thiamine·HCl, 2; pyridoxine·HCl, 2.5; biotin, 0.1; folic acid, 0.2; vitamin B_{12}, 0.01.

TABLE 1

Composition of experimental diets

Component	A	B	C
	gm/100 gm diet		
Casein	23.5	23.5	23.5
Sucrose	72.0	44.5	54.0
Cottonseed oil		15.0	
Celluflour[1]		12.5	10.0
Linoleic acid[2]			8.0
Salts IV[3]	4.0	4.0	4.0
Vitamin mix[4]	0.5	0.5	0.5

[1] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[2] Technical grade, Matheson Company, Norwood, Ohio; 55% linoleic acid, remainder similar chain length acids.
[3] Hegsted et al. ('41).
[4] See text.

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Animals receiving the semipurified diets were given vitamins A, D and E as α-tocopherol-fortified haliver oil supplement. Fluoride was incorporated into these diets in the form of anhydrous, reagent grade NaF.

Fecal samples for fat analysis were collected daily, stored at 0°C, and then dried in vacuo at 55°C for 20 hours. Total fecal fat was determined by the method of Van de Kammer et al. ('49). For differential lipid analysis, a 5-gm portion of dried feces was extracted with ether 24 hours in a Goldfisch apparatus to obtain the neutral fat and free fatty acids. After removal of the solvent, the residue was redissolved in petroleum ether[2] and titrated with 0.1 N sodium ethoxide to determine the amount of free fatty acids and then the neutral fat by difference. The soap-bound fatty acids in the residual feces were measured as described by Van de Kammer et al. ('49).

The amount of fatty acids liberated from Tween 20[3] upon incubation with the enzyme preparation was used as a lipase assay (Bier, '55). Fatty acids, mono-, di-, and triglycerides were separated on silicic acid (Mattson and Beck '55) and fluorine was determined by a modification[4] of the Willard and Winter ('33) method.

EXPERIMENTAL

Because of known irritation of epithelial tissue by fluorides, and possible changes in intestinal flora due to fluoride ingestion, a change in the level of metabolic fat was considered as a possible cause of the increased fecal fat excretion in fluorotic animals. Two groups of 6, 150-gm rats were given the stock diet or the same diet with 0.1% of NaF added (450 ppm F) for two weeks. At this time they were given the fat-free semipurified diet A (see table 1) with and without fluoride and supplemented with fortified haliver oil. After a three-day transition period, feces were collected for 9 days and total fecal fat determined. The fluoride-fed rats excreted 73 mg of fatty acid per gm of dry feces and the control animals, 52. The food intake of the fluorotic animals was less and hence the total fat excretion for the 9-day period was 204 mg for the fluoride-fed rats and 197 mg for the controls. The standard deviations were less than 20% of these mean values.

It was felt that an examination of the distribution of fecal lipids into neutral, free fatty acid and soap fractions would give an indication of the cause of the elevated fecal lipids in fluorotic animals. A relatively large excretion of neutral fat might indicate a lack of lipolytic action, whereas an impairment of absorption would result in a build up of hydrolytic products, including free fatty acids. Six weanling female rats were placed in each of three lots and fed as follows: diet B (see table 1) fed ad libitum; diet B plus 0.1% of NaF fed ad libitum; and diet B pair-fed to the fluorotic animals. Both the fluoride-fed, and the pair-fed control animals gained an average of 63 gm over a 4-week period, while the full-fed controls gained an average of 104 gm. The results of the analysis for fecal fat during the third week of the experiment are presented in table 2. The results of the first week were qualitatively similar, but the differences are not as great, as the full

[2] Skellysolve B, b. p. 60 to 68°C.

[3] Polyoxyethylene sorbitan monolaurate, Atlas Powder Company, Wilmington, Delaware.

[4] Alcoa Research Laboratory, Aluminum Company of America, Aluminum Research Laboratories, 1947, technical paper 914.

TABLE 2

Effect of fluoride ingestion on excretion of fecal lipids

	Percentage of ingested fat excreted		
	Control group, fed ad libitum	Control group, pair-fed	Experimental, fed 0.1% NaF
	%	%	%
As neutral fat	1.46 ± 0.15[1]	1.42 ± 0.06	5.18 ± 1.61
As free fatty acid	1.85 ± 0.23	1.74 ± 0.20	3.22 ± 0.43
As soap-bound fatty acid	3.00 ± 0.78	1.91 ± 0.36	6.34 ± 1.21
As total fecal lipid	6.31 ± 1.06	5.07 ± 0.36	14.74 ± 2.30

[1] Standard deviation (6 rats/group).

effect of the fluoride ingestion had not yet been realized. The total amount of the ingested lipid found in the feces of the animals was about 15% for the fluorotic animals and 6 and 5% for the control ad libitum- and pair-fed groups, respectively. There was a fourfold increase in the excretion of neutral fat in the fluorotic animals, and a two- to threefold increase in the excretion of free and soap-bound fatty acids. The differences between the two control groups were due almost entirely to an increase in the soap-bound fatty acids in the group fed ad libitum.

Although dietary lipids are by no means completely hydrolyzed in the intestine, free fatty acids are one of the major end products of fat digestion (Skipski et al., '59). Therefore, it was of interest to determine whether fluorotic animals could utilize free fatty acids as a sole lipid source.

Two groups of 6 weanling rats each were given an 8% linoleic acid diet (diet C, table 1) with and without 0.1% of NaF. After two weeks a 7-day fecal collection was made and the total fecal fatty acids determined. The results of the analysis showed that the control animals excreted 7.8 ± 0.5% (mean ± standard deviation) of the ingested lipid and the fluoride-fed rats only 4.8 ± 0.8%.

If fluoride exerted a direct effect on intestinal lipase activity, the presence of fluoride in the gut would be required for the maximum effect on fecal fat excretion to be observed. An experiment was set up to study the effect of administration of fluoride by intraperitoneal injection or by stomach intubation.

Two groups of 6, 28-day-old rats each were given the 15% fat diet B (table 1). One group was given by stomach tube 3 mg of fluoride per day (as NaF) in 2 ml of physiologic saline. This dose was divided and given each morning and evening, while the control group received an equal amount of physiologic saline. Six days after the first fluoride administration, a 6-day fecal collection was started. At the end of this period the fluoride administration was changed to intraperitoneal injection and after a two-day transition period another 6-day fecal collection was begun. Following this, the fluoride intake was raised to 4 mg per day, and the same pattern of stomach intubation for 8 days followed by intraperitoneal administration for 8 days was continued. The fecal samples were analyzed for the different lipid fractions as previously described and the results are presented in table 3.

There was considerable variation of lipid excretion by the control animals over the 4 collection periods. However, when each group was compared with the corresponding fluoride-fed animals it was apparent that in all cases the administration of fluoride, regardless of level or mode of administration, increased the fecal lipid excretion. The data indicate that the in-

TABLE 3

Effect of mode of administration of fluoride on distribution of fecal lipids

	Percentage of ingested fat excreted			
	3 mg F/day by stomach tube	3 mg F/day intraperitoneally	4 mg F/day by stomach tube	4 mg F/day intraperitoneally
	%	%	%	%
	Fluoride administered			
As neutral fat	4.37 ± 1.30[1]	3.48 ± 0.82	2.53 ± 0.29	1.87 ± 0.29
As free fatty acids	2.00 ± 0.13	1.82 ± 0.26	1.36 ± 0.09	1.00 ± 0.12
As fatty acid soaps	2.06 ± 0.42	2.98 ± 0.45	4.08 ± 0.73	3.40 ± 0.29
Total lipids	8.43 ± 1.31	8.28 ± 1.17	7.97 ± 0.48	6.27 ± 0.64
	Controls			
As neutral fat	2.37 ± 0.49[1]	2.16 ± 0.36	1.68 ± 0.75	1.61 ± 0.21
As free fatty acids	1.32 ± 0.16	1.48 ± 0.11	0.98 ± 0.15	0.78 ± 0.12
As fatty acid soaps	3.16 ± 0.51	3.41 ± 0.57	3.19 ± 0.42	2.91 ± 0.50
Total lipids	6.85 ± 1.05	7.05 ± 0.59	5.85 ± 0.57	5.30 ± 0.63

[1] Standard deviation (6 rats/group).

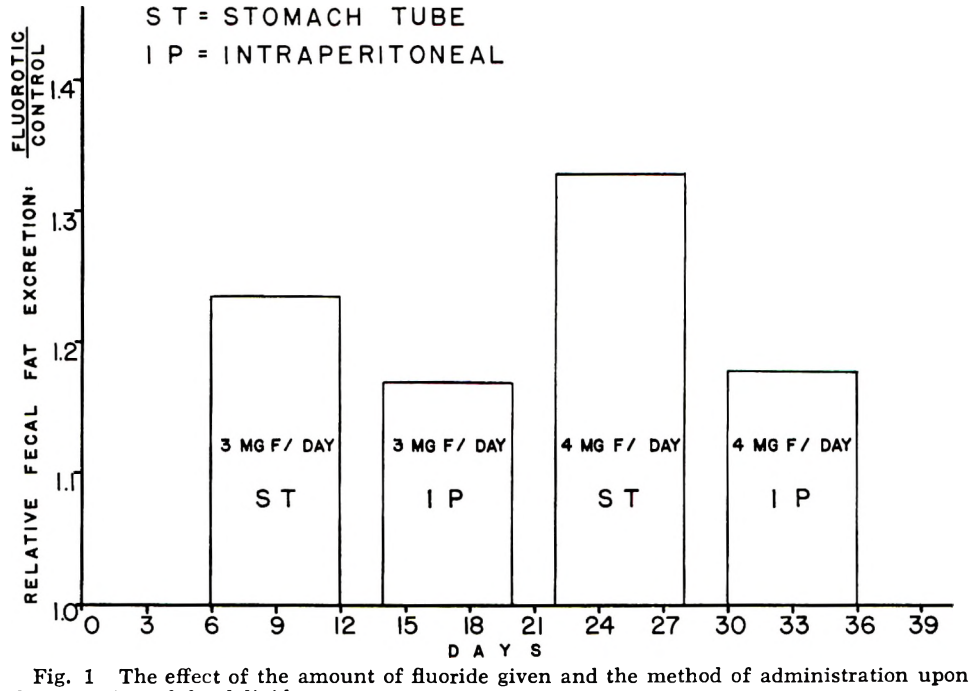

Fig. 1 The effect of the amount of fluoride given and the method of administration upon the excretion of fecal lipids.

crease of fecal lipids was of greater significance during the intubation period than when the animals were receiving fluoride by intraperitoneal injection. This relationship is better expressed in figure 1 where the relative excretion values are presented. It was found that rats given either 3 or 4 mg of fluorine per day by stomach tube had about 300 ppm of F in the dried feces, whereas the animals receiving 3 and 4 mg of fluorine per day by intraperitoneal injection excreted about 100 and 200 ppm of F, respectively, in the feces. The feces from rats receiving the control diet contained about 20 ppm of F. The fluoride in the gut of the injected animals could presumably arise by free diffusion from the peritoneal cavity, by bile excretion, or by pancreatic, gastric or intestinal secretion.

Because of indications from the previous experiments that fluoride might be influencing lipase activity, attempts were made to demonstrate such an inhibition.

Although it appeared rather unlikely that there would be any influence of fluoride on the secreting organ, a study was made of the level of pancreatic lipase. The rats used had been receiving the stock diet or this diet with 0.1% of added NaF for two weeks. Prior to this time they had received a semipurified diet with the same amount of fluoride. Acetone powders were prepared from the pancreatic tissues of 6 animals in each lot, and lipase was extracted with 50% glycerol for analysis. There was no difference in the mean lipase activity of the two lots, but considerable variation within each group.

A more likely point of inhibition would be in the intestinal lumen. Rats which previously had been used in the metabolic lipid study were fed the stock diet with and without 0.1% NaF added for two weeks, then killed by a blow on the head, and 20 cm of the small intestine beginning 2 cm below the plyoric sphincter tied off. The first and third 5-cm sections were slit open and added to 4 ml of the substrate, stirred for one minute and removed. After this the enzyme activity was determined in the usual manner. The second and 4th 5-cm sections were treated in the same way in water and titrated for a blank value. Rats which had been receiving 4 mg of fluorine per day by interperitoneal injection were treated in the same manner. The results

TABLE 4

Effect of fluoride on lipase activity in intestinal lumen

	Lipase activity[1]	
	Control	Fluoride-fed
Stock diet, 0.1% NaF given ad libitum	1.26 ± 0.26[2]	0.94 ± 0.29
Stock diet, 4 mg F/day given intraperitoneally	1.11 ± 0.20	1.06 ± 0.27

[1] Milliliter base/10 min/10 cm intestine.
[2] Standard deviation (6 rats/group).

of these determinations are presented in table 4. The drop in lipase activity evident in the fluoride-fed lot was not statistically significant (P between 0.1 and 0.15). However, in 5 of the 6 pairs of rats there was a decrease of from 20 to 50% lipase activity in the fluorotic animal.

Although the measure of lipase activity is liberation of fatty acid, a large portion of the triglyceride is not completely degraded. Present evidence would indicate that within the lumen of the intestine after lipase action there is a mixture of free fatty acids, mono-, di-, and triglycerides. An attempt was made to see whether this distribution was changed in fluorotic animals.

Weanling rats were given the 0.1% NaF stock diet. Control animals were given only enough of the stock diet to maintain their weight at the same level as the fluoride-fed rats. The rats were used when they weighed about 180 gm. After a 48-hour fast, the rats were given 1 ml of olive oil by stomach tube and three hours later were killed, the intestinal lipids isolated as described by Skipski et al. ('59) and the composition of the isolated lipids determined.

Recoveries from the column were within 5% of the amount added and separation was checked with mixtures of known composition.[5] The results of these analyses are presented in table 5. There was no difference in the character of the lipid isolated from the two groups. Attempts to study this distribution in rats which were given fluoride by stomach tube at the same time as the olive oil, failed. The fluoride

TABLE 5

Distribution of intestinal lipids of fluorotic and control rats

	Percentage of total lipids	
	Control	Fluorotic
	%	%
As free fatty acids[1]	35.0 ± 4.7[2]	31.8 ± 4.2
As neutral fat	29.8 ± 4.0	33.6 ± 4.9
As diglycerides	24.8 ± 3.3	25.0 ± 3.3
As monoglycerides	10.4 ± 1.5	9.6 ± 1.3

[1] Expressed as palmitic acid.
[2] Standard deviation (5 rats/group).

delayed the emptying of the stomach and made it difficult to recover a representative sample of lipid.

DISCUSSION

The lack of effect of fluoride on fecal fat excretion, using a fat-free diet, would seem to eliminate the possibility that an increase in metabolic fat was responsible for the apparent poor utilization of dietary fat in the fluorotic rat. Neither does the evidence presented here support an interference with absorption as being the cause. The fluorotic rats were able to utilize free fatty acids in the diet very efficiently, and the distribution of the end products of lipid hydrolyses in the gut was not altered in the fluorotic animals.

The data presented are consistent with the hypothesis that there is a partial inhibition of lipase within the gut of the fluoride-fed rat. This is supported by the relatively greater increase of the neutral fat fraction of the fecal lipid in the fluorotic animals, and by the more pronounced effects on fecal lipid excretion when fluoride was administered by stomach intubation than by intraperitoneal injection. Attempts to measure intestinal lipase activity were complicated by dilution of the fluoride concentration in the assay system. However, some inhibition of lipase was observed even though it was estimated that the fluoride in concentration in the intubation mixture was no greater than 20% of that which had been observed in the intestinal fluid.

[5] Pure samples of 1-monopalmitin and 1,3-dipalmitin were supplied by Dr. F. H. Mattson, Procter and Gamble Company.

SUMMARY

The experiments described were carried out as attempts to determine the cause of the high fecal fat excretion previously observed in fluorotic rats.

Fluoride ingestion had no effect on the level of metabolic fat. Although all fractions were elevated in the fluorotic rats, the neutral portion of the fecal lipid was raised to a greater extent than free or soap-bound fatty acids. Dietary free fatty acids were efficiently utilized by fluoride-fed rats. When the fluoride intake was equalized, more fat was observed in the feces of animals receiving fluoride by stomach intubation than by intraperitoneal injection. There was an indication of a lowered lipase activity in the intestine of rats receiving fluoride in the diet, but not in rats given fluoride intraperitoneally. Under the experimental conditions studied, the distribution of the end products of lipolysis was not effected by fluoride ingestion.

It was concluded on the basis of these results that the high level of fecal lipid in fluorotic animals can be explained in part on the basis of a partial inhibition of lipase activity in the intestine.

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Vitamin Absorption Studies

I. FACTORS INFLUENCING THE EXCRETION OF ORAL TEST DOSES OF THIAMINE AND RIBOFLAVIN BY HUMAN SUBJECTS

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Although many workers have studied factors influencing the excretion of thiamine and riboflavin by human subjects, very little is known of the effects of size of dose on the urinary excretion of these vitamins. Melnick et al. ('45) stated that, in normal subjects receiving nutritionally adequate diets, the urinary excretion of the water-soluble vitamins, or their derivatives, was directly proportional to the quantity consumed. For thiamine and riboflavin, the upper limits tested by Melnick et al. were 7.5 and 10 mg, respectively; and inspection of their data reveals that a break in the linear dose-response relationship occurred at an intake of thiamine of about 5 mg from the test dose. Friedemann et al. ('48) concluded that the intestinal absorption of thiamine, and hence its excretion, was extremely limited. The maximum amount which could be taken orally without a resultant increase of fecal thiamine was about 5 mg per day. Previously, Schultz et al. ('38) had reported that, in subjects receiving 5 mg of thiamine daily, almost all of an additional oral dose of 5 mg thiamine was recovered in the feces.

Also, the effects of mode of administration on the urinary excretion and utilization of doses of thiamine and riboflavin have not been studied extensively. Horwitt et al. ('50) concluded that daily administration of three, 2-mg doses of riboflavin was no more effective in treatment of ariboflavinosis than a single 6-mg dose. Chapman and Campbell ('55) and Morrison et al. ('60b) observed that the total urinary excretion of riboflavin was not affected by giving the total dose in a number of small doses over a period of several hours. In animal studies bearing on this problem, Sarett and Morrison ('60) found that, in growing rats, the utilization of B-vitamins was similar if the vitamins were given in the diet, or once daily by stomach tube.

Since there is a great deal of interest in the possibility of modifying the rate of absorption to produce sustained or delayed effects of vitamins, it is important that fundamental information be available on factors influencing absorption and excretion rates. The studies reported herein were conducted to study systematically the effects of size of dose and mode of administration on the urinary excretion of thiamine and riboflavin.

EXPERIMENTAL

Six normal male subjects, known to be receiving nutritionally adequate diets, were used in the experiments. During the tests, they continued to eat their normal diets, but were cautioned to consume meals similar in composition from day to day, and to avoid eating foods high in thiamine or riboflavin. The subjects received doses of one to 20 mg of thiamine or riboflavin in solution or in tablets known to disintegrate rapidly, at 8:45 A.M. after breakfast. It should be emphasized that the vitamins were not given together. Urine was collected in opaque bottles containing 2 ml of 3.5 N H_2SO_4 for the riboflavin studies, or of glacial acetic acid for the thiamine studies, after two, 4, 6, 8, 14 and 24 hours, and at 4-hour intervals thereafter until the rate of excretion approached blank values. The amounts of thiamine and riboflavin obtained in the urine after dosing were corrected by subtracting the appropriate blank

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value determined on the urine of the same subjects. Urinary thiamine was determined by the thiochrome method of Mickelsen et al. ('45), and urinary riboflavin was determined by the U.S.P. XV fluorometric procedure ('55). Curves of net rate of excretion were plotted on semi-logarithmic paper, as suggested by Swintosky et al. ('57).

In studies on divided doses of thiamine, the subjects received 4 doses of 2.5 mg, or three doses of 2.5, 2.5 and 1.0 mg, at two-hour intervals, beginning at 8:45 A.M. In similar studies on riboflavin, 4 doses of 2, 1.5, 1.5 and 1 mg of riboflavin were given at two-hour intervals, beginning at 8:45 A.M. Urine was then collected in the same manner as for the single test doses.

Melnick et al. ('39) reported that thiamine given three hours before breakfast was excreted to a much less extent than the same test dose given with the largest meal of the day. To study this problem further, doses of 2.5 mg of thiamine and 5.0 mg of riboflavin were given at 6:15 A.M., one hour before breakfast. Rates of urinary excretion of the two vitamins were compared with those found when the vitamins were given at 8:45 A.M. after breakfast.

In view of the current interest in sustained-release vitamin preparations, the availability of thiamine in the products examined by Morrison et al. ('60b) was determined. Net urinary excretion of thiamine after ingestion of the products was compared with that obtained after ingestion of standard doses of the vitamin. Thiamine in the products was determined by the thiochrome procedure (Mickelsen et al., '45).

The data of the experiments were analyzed by appropriate statistical procedures (Snedecor, '55).

RESULTS AND DISCUSSION

Data on blank excretion of riboflavin were presented previously (Morrison et al., '60a). In brief, the mean daily excretion varied from 758 to 1539 μg, and averaged 1193 μg. Blank excretion of thiamine averaged 539 μg daily, with a range of from 413 to 892 μg. These values, which are all well within the "normal" range (ICNND, '57), indicate that the subjects were receiving diets containing adequate amounts of the vitamins tested.

Effect of size of dose. Riboflavin excretion, expressed as percentage of the dose, remained relatively constant for dosages varying from 1.0 to 20.0 mg (table 1 and fig. 1). These results are in agreement with those of Melnick et al. ('45) and Morrison et al. ('59). Chapman and Campbell ('55) also found that the percentage of riboflavin excretion from doses of 5 to 10 mg was relatively constant. The lower percentage excretion which they observed from smaller doses may have been due, in part, to the fact that at low doses the blank excretion exceeds that from the dose, and variability is increased. Percentage of thiamine excretion, however, was significantly influenced by size of the dose, and decreased markedly with doses greater than 2.5 mg (table 1 and fig. 1). Increasing the dose from 2.5 mg to 20.0 mg increased the amount of thiamine excreted by only approximately 0.2 mg.

In figures 2 and 3, the net rates of excretion of thiamine and riboflavin, respectively, were plotted against time, on a semi-logarithmic plot. Standard errors for each time point are also shown. As might be expected, variation was somewhat greater for the smaller doses. The peak excretion rate of thiamine rose slowly as the dose was increased from one to 20 mg, whereas that for riboflavin rose much more rapidly as

TABLE 1

Effect of size of dose on urinary excretion of thiamine and riboflavin

Dose	Thiamine, mean net excretion		Riboflavin, mean net excretion	
mg	*mg*	*% of dose*	*mg*	*% of dose*
1	0.276	27.6 ± 4.3[1]	0.625	62.5 ± 10.7
2.5	0.525	21.0 ± 3.6	1.556	62.2 ± 5.1
5.0	0.435	8.7 ± 0.7	2.960	59.2 ± 4.8
10	0.640	6.4 ± 0.6	5.800	58.0 ± 4.2
20	0.740	3.7 ± 1.1	12.680	63.4 ± 11.5

[1] Standard error of the mean.

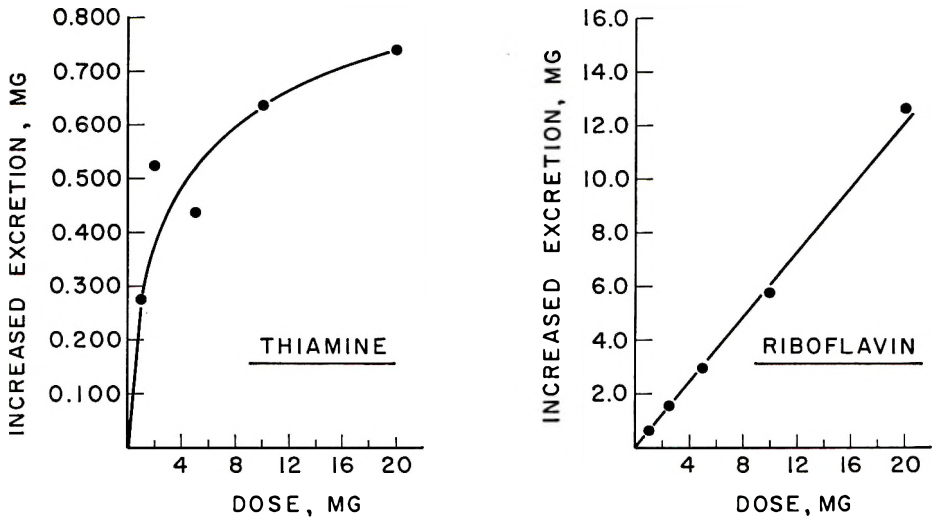

Fig. 1 Effect of dosage level on urinary excretion of thiamine and riboflavin.

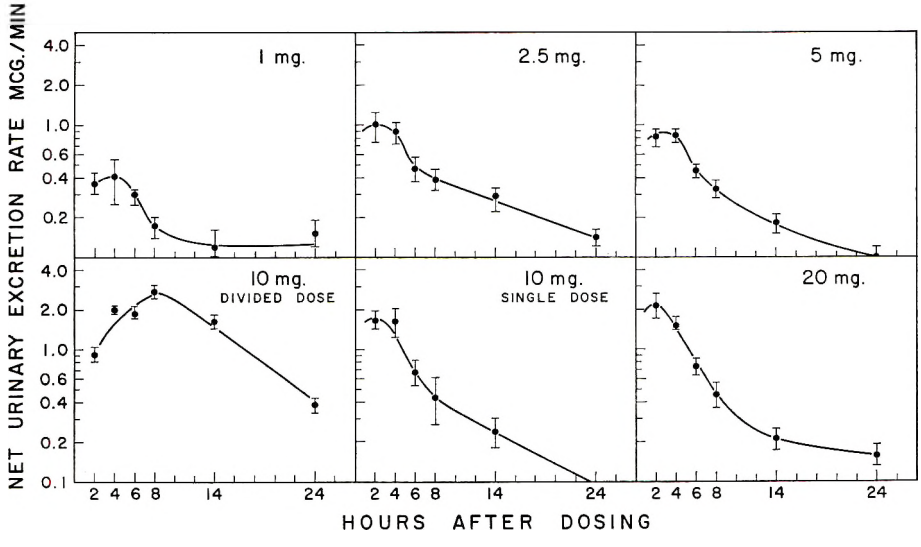

Fig. 2 Effect of size and mode of administration of dose on rate of urinary excretion of thiamine.

the dose was increased. The peaks of excretion occurred approximately two hours after administration of the doses, suggesting that the vitamins are absorbed high in the intestinal tract. The biological half-life (Swintosky et al., '57) of both thiamine and riboflavin as determined from urinary excretion rates after giving single doses of the vitamins, varied from two to 4 hours.

Effects of mode of administration of dose. The effects of divided doses on the excretion of thiamine and riboflavin are summarized in figures 2 and 3, respectively. The values shown for each vitamin represent the average of two trials, which were in close agreement. Although the results of both studies with divided doses of thiamine agreed closely, only the findings with the 10-mg dose are shown in figure 2. The urinary excretion of thiamine was markedly increased by giving the vitamin in divided doses at two-hour intervals. The excretion from the 10-mg dose was in-

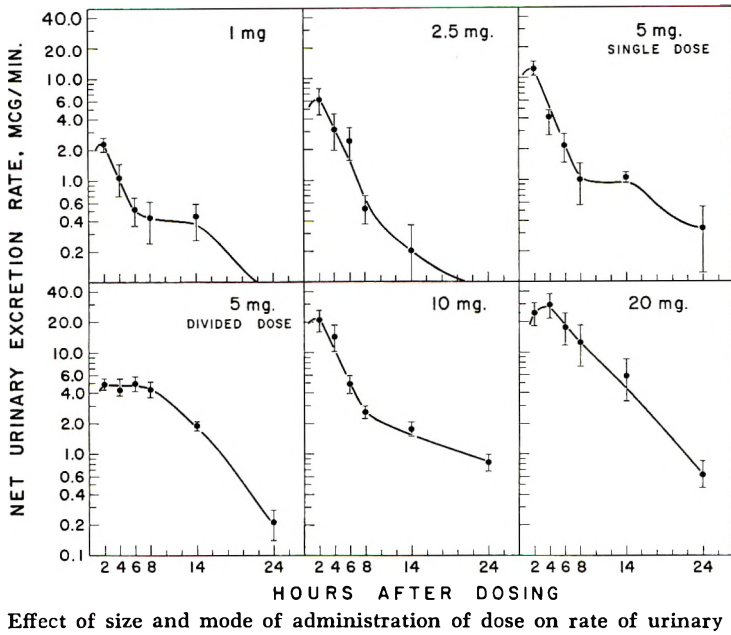

Fig. 3 Effect of size and mode of administration of dose on rate of urinary excretion of riboflavin.

creased from 6.1 to 17.6% by dividing the dose into four 2.5-mg aliquots. From the dose-response relationship shown in figure 1, it can be calculated that the percentage excretion from a single 6-mg dose of thiamine would be approximately 8 to 9%, whereas that from the 6-mg divided dose was found to be 20.3%. With the exception of the first 4 hours, the excretions from the divided doses were significantly greater than from the single doses at all time points tested. It is of interest to note that Chow et al. ('58) have observed that large amounts of vitamin B_{12} were also more efficiently absorbed if given in divided doses.

Since the percentage of riboflavin excretion was not significantly influenced by the dosage level, the values for the 6-mg divided dose were converted to the basis of a 5-mg dose, to be directly comparable with the 5-mg single dose. As shown in figure 3, the overall excretion of riboflavin was not significantly influenced by giving the total dose in several small doses. These results agree with those of Chapman and Campbell ('55) and Morrison et al. ('60b). Administration of riboflavin according to the divided dosage schedule gave sustained urinary excretion of the vitamin for approximately 8 hours, and prevented the high peak and subsequent rapid decline in urinary excretion obtained with the single dose of riboflavin.

The excretion values obtained when the test doses of thiamine and riboflavin were taken one hour before breakfast indicated that excretion of riboflavin under these circumstances was similar to that obtained when the vitamin was given at 8:45 A.M., after breakfast. Excretion of thiamine, however, was somewhat less (16.8% compared with 21.0%) when the vitamin was given before breakfast, although the difference was not statistically significant ($P = 0.05$).

Data on the availability of thiamine in the sustained-release products examined are summarized in table 2. Since the percentage of thiamine excretion was markedly influenced by the size of the dose and by dividing the dose, it was necessary to determine the appropriate standard with which to compare the various products. It would appear that, to be fully available, sustained-release thiamine preparations should give the same percentage excretion values as those found with similar amounts of thiamine given in divided doses. The studies with divided doses of thiamine

TABLE 2

Physiological availability of thiamine in sustained release preparations

Product	Thiamine	Urinary thiamine excretion		Physiological availability
		Product	Standard[1]	
	mg/unit	*% of dose*	*% of dose*	*%*
A	9.0	7.2	14.7	49.0
B	21.7	2.1	8.1	25.9
C	15.4	1.8	10.3	17.5
D	6.6	6.8	18.0	37.8
E	15.0	6.1	10.4	58.7

[1] Size of standard dose equal to one third that of the product.

showed that excretion from a large divided dose was similar to that from the aliquots in which it was given. For instance, the excretion from the 10-mg divided dose, given as four 2.5-mg aliquots, was 17.6%, not significantly different than that from a single 2.5-mg dose (21.0%). Sustained-release products are designed to release one third of the total dose immediately, and the remaining two-thirds over a period of approximately 10 hours. It would seem logical, therefore, to compare the excretion from a product with that from a dose of the standard equivalent to one third of the total dose in the product, as determined by analysis. According to this procedure, to show full availability, the percentage excretion from a sustained-release product containing 6 mg of thiamine should be similar to that from a single 2-mg dose of thiamine in solution. Excretion from the various doses of standard was calculated from the dose-response relationship shown in figure 1. The results (table 2) indicated that none of the products gave full availability or showed evidence of sustained release properties.

It might be argued that the appropriate standard with which to compare the various products would be a single standard, rather than a divided standard. Using this procedure, the values for percentage availability of the products were as follows: A, 103; B, 55; C, 38; D, 71; and E, 130. Thus, even when tested in this manner, three of the products were unavailable, and, with the posssible exception of product E, none exhibited sustained-release properties. Morrison et al. ('60b) previously found low availability and lack of sustained release for these products as judged by urinary excretion of riboflavin. Thus, although it is possible to increase thiamine absorption by repeated doses, there is no convincing evidence that such can be obtained from commercially available single dosage forms.

The findings of these studies, along with those of Schultz et al. ('38) and Friedemann et al. ('48) suggest a definite barrier to intestinal absorption of thiamine. The results suggest, furthermore, that single oral doses of thiamine above 2.5 to 5.0 mg are largely wasted. There would appear to be little advantage to the common practice of administering large amounts of thiamine in single oral doses. If high blood thiamine concentrations are desired, it would seem advisable to administer the vitamin parenterally.

It is of interest to note the apparent difference in absorption of thiamine and riboflavin, as judged by urinary excretion. Although both vitamins are normal and essential constituents of every cell, are required in approximately the same amounts in the diet, and are readily phosphorylated, the results indicated the evident existence of an effective intestinal barrier to thiamine absorption. The findings suggest, but do not prove, that riboflavin absorption may occur by passive transfer, without the necessity for a special mechanism, but that thiamine absorption involves active transport. Studies to determine the mechanism of absorption of thiamine and riboflavin are now in progress.

SUMMARY

Normal male subjects, receiving nutritionally adequate diets, were given oral doses of one to 20 mg of thiamine and riboflavin and the urinary excretion of the vitamins was determined until excretion levels returned to those found prior to dosing. The excretion of thiamine, expressed

as percentage of dose, decreased markedly with doses greater than 2.5 mg. Increasing the dose from 2.5 to 20 mg increased the amount of thiamine excreted by only 0.2 mg. In contrast, riboflavin excretion remained a constant percentage of dose. Excretion of thiamine after a 10-mg dose was increased threefold by giving the vitamin in 4 doses of 2.5 mg each, at two-hour intervals. Preparations designed to produce sustained release of thiamine in a single dosage form were found to vary markedly in availability and showed no evidence of sustained-release properties. Administration of riboflavin in divided doses produced sustained urinary excretion of the vitamin, but had no significant effect on the total amount of riboflavin excreted.

ACKNOWLEDGMENTS

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The Weight Gain and Feed Intake of Chicks Fed a Ration Diluted with Cellulose or Kaolin

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The literature pertaining to the effects of the dilution of poultry diets is primarily concerned with experiments in which a relatively inert material was used to replace a more available ration component. This procedure changes the relative proportions of the individual nutrients within the diet and provides little information regarding the properties of the diluent *per se*. There is a scarcity of information concerning the effects of including an inert diluent at the expense of a portion of the entire diet. This latter technique, assuming the diluent to be nutritionally unavailable, should lower the concentrations of the nutrients in the feed without changing their relative proportions. Experiments employing this latter method of dilution should provide useful information not only regarding the properties of the diluents but also concerning the importance of dietary nutrient concentration.

Hill and Dansky ('54) reported that when 10% increments of a 20% protein chick diet were replaced with oat hulls, up to a maximum of 50%, growth and energy consumption decreased while feed consumption increased, although the increased consumption was not sufficient to compensate for the reduced energy content of the diets. An exception to these findings may be noted in the data relating to the treatment group receiving a ration composed of 90% basal diet plus 10% oat hulls; the birds of this group made slightly larger weight gains and consumed more energy than the birds of the control group which were fed the basal diet with no added oat hulls. A comparison of the data obtained when dilution by whole ration replacement was employed with that obtained when dilution was achieved by replacing corn and wheat with oat hulls led the authors to conclude that protein rather than energy was the limiting deficiency. The energy values used were based on the productive energy data of Fraps ('46).

Rand et al. ('56) fed chicks purified diets of varying protein content each supplemented with zero, 10 and 19% of added fiber. No differences in weight gains could be detected between the birds fed the control diets and those receiving the control diets plus 10% of fiber. Nineteen per cent of added fiber reduced weight gains. These findings support those of Hill and Dansky ('54) who demonstrated no depression in growth until oat hulls in excess of 10% were included in the ration at the expense of the basal portion.

Mraz et al. ('57) formulated a series of diets differing in calculated productive energy content. The densities of the diets were varied, by interchanging sand and vermiculite, allowing the authors to study the importance of dietary energy concentration both in terms of calories per unit of weight and calories per unit of volume. The latter measure appeared to be the most useful and it was reported that whereas a productive energy concentration of 0.75 Cal./cm^3 was adequate for the maintenance of rapid growth, 0.72 Cal./cm^3 was indequate.

The present report concerns an experiment designed to study the influences of cellulose and kaolin upon the feed consumption and weight gains of chicks.

EXPERIMENTAL

The experimental design was that of a randomized block with 4 replicates. Day-old chicks were placed in battery brooders where they were fed the basal diet (table 1) ad libitum. When the birds were two weeks and 4 days old they were weighed, divided into groups having a weight spread

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of 5 gm, and then distributed among 60 pens until each pen contained 14 birds (7 ♂ + 7 ♀ per pen in replicate 1, and 8 ♂ + 6 ♀ in replicates 2, 3 and 4). Each of the 15 experimental diets (table 2) was then randomly assigned to 4 pens. The diet treatments were designed so that increasing amounts of the basal diet (zero, 6, 12, 18, 24, 30, 36 and 42%) were replaced with non-nutritive cellulose,[1] and with kaolin. Following a three-day ration adjustment period the birds entered upon a 7-day experimental period during which weight changes and feed consumption, on a per pen basis, were recorded.

TABLE 1

Composition of the basal diet

	pounds
Ground corn (fine)	36
Ground wheat (fine)	15
Soybean oil meal (44% protein)	32
Stabilized tallow	5
Dehydrated alfalfa meal (17% protein)	2
Meat meal (50% protein)	2.5
Fish meal (65% protein)	2.5
Dried whey product (55% lactose)	2.5
Ground limestone	1.5
Dicalcium phosphate	0.5
Iodized salt (0.015% KI)	0.25
	gm
Manganese sulphate (75%)	9.0
Zinc oxide (80%)	3.0
Vitamin A supplement (10,000 IU/gm)	22.7
Vitamin D_3 supplement (1,500 ICU/gm)	22.7
DL-Methionine (feed grade)	22.7
Riboflavin (1 gm/oz)	5.0
D-Ca pantothenate (2 gm/oz)	5.0
Niacin	0.9
Procaine penicillin G (10 gm/pound)	22.7
Vitamin B_{12} supplement (9 mg/pound)	34.1
Choline chloride (25%)	34.1
3-Nitro, 4-hydroxy-phenylarsonic acid (10%)	22.7

Metabolizable energy (ME) values were obtained for the diets and have been discussed in an earlier report (Sibbald et al., '61a). Ration density values were obtained by two procedures. The first method which employed a device used for the determination of bushel weights of grains yielded density data expressed in terms of grams per milliliter of dry feed. These data (table 2) were considered to be unsatisfactory; not only were they difficult to reproduce but is was also observed that, as the level of dilution with kaolin (diets 9 to 15) increased, the changes in ration density were not linear. The second procedure involved placing 50 gm of feed into a 250-ml volumetric flask, the addition of water from a burette to bring to volume, and frequent shaking to minimize the displacement of water by air. The resulting data (table 2),

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Composition and analysis of the experimental diets

Diet	Basal diet[1]	Cellulose[2]	Kaolin	Protein[3]	ME[4]	Density (dry basis)	Density (water displacement)
	%	%	%	%	*Cal./gm*	*gm/ml*	*gm/ml*
1	100	—	—	23.5	2.95	0.574	1.416
2	94	6	—	22.8	2.80	0.546	1.425
3	88	12	—	20.7	2.63	0.521	1.437
4	82	18	—	19.5	2.44	0.505	1.445
5	76	24	—	17.6	2.30	0.458	1.458
6	70	30	—	16.5	2.11	0.439	1.464
7	64	36	—	15.1	1.91	0.411	1.475
8	58	42	—	13.9	1.76	0.378	1.482
9	94	—	6	22.0	2.79	0.622	1.484
10	88	—	12	20.4	2.61	0.695	1.544
11	82	—	18	18.9	2.43	0.677	1.611
12	76	—	24	17.7	2.30	0.658	1.636
13	70	—	30	15.4	2.18	0.625	1.695
14	64	—	36	15.2	1.97	0.588	1.730
15	58	—	42	13.9	1.82	0.676	1.773

[1] Composition of basal diet presented in table 1.
[2] Non-nutritive cellulose, Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Determined by the method of Kjeldahl (A.O.A.C., '55).
[4] Determined by the method described by Sibbald et al. ('61b).

expressed as the weight of feed required to displace 1 ml of water, were considered to be better measures of ration density than the data obtained by the original procedure, for they were highly reproducible and varied in a linear manner with the level of either the cellulose or kaolin in the diets. It is of interest to note that as the level of cellulose in the diets increased, the dry densities of the feeds decreased, whereas the densities measured by water displacement increased. Obviously the type of density values used would have a profound influence upon the interpretation of the experimental data. For the reasons stated previously and because feed becomes wet once it enters the crop of the chicken, the water displacement density values were used in the interpretation of the data resulting from this experiment.

The weight change and feed consumption data, though recorded on a per pen basis, were reduced to a per bird basis in order to simplify the handling of the data in subsequent statistical analyses. The statistical treatments were based on methods described by Snedecor ('56). To facilitate the interpretation of the results, data from diet groups 1 to 8 (zero to 42% of cellulose) and groups 1 plus 9 to 15, (zero to 42% of kaolin) were sometimes analyzed separately. When the observations from all treatments were pooled the data resulting from diet treatment 1 (100% basal) were employed only once.

RESULTS AND DISCUSSION

The weight gain and feed consumption data have been summarized and are presented in the form of means in table 3. The data show that the replacement of 6% of the basal diet with non-nutritive cellulose resulted in only a very small decrease in weight gain but that at a replacement of 12% or more the weight gain was considerably less than that of the birds fed the basal diet with no diluent. The birds receiving the diets diluted with kaolin consistently made greater weight gains than the birds receiving an equivalent level of the cellulose and showed no reduction in weight gain at a dilution of 12%. These data suggest that chicks are better able to deal with diets diluted with kaolin than with diets extended with the cellulose.

TABLE 3

Mean weight gain and feed consumption data expressed on a per bird basis

Treatment		Average weight[1]		Weight gain		Feed consumed		Feed consumed		Basal consumed		Protein consumed		ME consumed	
Basal	Diluent	C	K	C	K	C	K	C	K	C	K	C	K	C	K
%	%	*gm*		*gm*		*gm*		*ml*		*gm*		*gm*		*Cal.*	
100	—	294		100		224		158		224		53		661	
94	6	286	293	98	102	234	247	164	166	220	228	53	54	655	689
88	12	273	290	91	100	239	250	166	162	210	220	49	51	628	652
82	18	259	278	80	88	237	251	164	156	194	206	46	47	578	610
76	24	249	268	63	85	234	268	160	164	178	203	41	47	538	616
70	30	232	258	51	67	219	260	150	153	153	182	36	40	462	567
64	36	210	250	31	62	197	279	134	161	126	178	30	42	376	550
58	42	199	241	20	54	190	296	128	167	110	172	26	41	334	539

[1] C refers to diets diluted with non-nutritive cellulose (Alphacel), K to diets diluted with kaolin.

Analysis of variance supported this interpretation; the weight gains of the birds receiving diets 2 to 8 (6 to 42% of cellulose) being significantly less ($P < 0.01$) than those fed diets 9 to 15 (6 to 42% of kaolin).

The weight of feed consumed by the birds receiving the diets diluted with the cellulose increased as the level of diluent increased, up to a maximum of 12% dilution, and then decreased as the degree of dilution increased. These data suggest that the birds attempted to compensate for the reduced nutrient content of the diet by increasing feed intake. That the birds were unable to do this when the diets contained more than 6% of cellulose is demonstrated by the data pertaining to the basal diet, protein and ME consumption. The groups receiving the kaolin-diluted diets, with one exception, increased the weight of feed consumed as the level of diluent increased, again indicating an attempt to maintain nutrient intake. When the level of diluent in the diet exceeded 12% the intakes of basal diet, protein and ME decreased rapidly though not to the same degree as when cellulose was the diluent. It is apparent that the variations in the weight gain data, associated with the types and levels of diluents, are reflections of the variations in nutrient intake.

The data pertaining to the volume of feed consumed are of interest not only because they reflect the attempts of the birds to compensate for the reduced nutrient concentration of the diets by increased feed intake but also because they appear to be associated with the average body weights of the chicks. Regression analysis of the volume of feed consumed (Y) and the average chick weight (X) data yielded the highly significant ($P < 0.01$) correlation coefficient: $r = 0.687$ at 58 degrees of freedom. This indicates that the volume of feed consumed was largely regulated by the body weights of the birds. As the diluents appeared to inhibit weight gain in treatment groups 3 to 8 (12 to 42% of cellulose) and groups 11 to 15 (18 to 42% of kaolin) only, the analysis was repeated using the data from these treatment groups alone. The correlation coefficient ($r = 0.763$ at 42 d.f.) was larger than that obtained when all the data were employed (0.687), and again proved to be highly significant ($P < 0.01$). The regression equation resulting from this latter analysis was

$$Y = 42 + 0.4578X$$

where Y is the volume of feed consumed (ml) per bird per week, and X is the average weight per bird during the week. The relationship between the volume of feed consumed and body weight helps to explain why the birds receiving the kaolin-diluted diets made greater weight gains than those receiving diets containing the non-nutritive cellulose. Kaolin and cellulose had densities of 2.500 and 1.566 gm/ml respectively; consequently, a basal diet diluted with the cellulose would contain less nutrients per unit volume than when diluted with the same percentage by weight of kaolin. Therefore, when the volume of feed consumed is a limiting factor, birds receiving a kaolin-diluted feed would consume more nutrients than those receiving feed containing an equivalent proportion by weight of the cellulose. This difference in nutrient intake would influence the body weight gains of the birds.

The foregoing explanation tends to oversimplify the effects of ration diluents on the weight gains of growing chicks. The intake of nutrients from diluted feeds is probably reduced initially primarily because the birds are unable to consume a sufficient volume of diluted feed to compensate for its reduced nutrient concentration. However, such factors as palatability and alterations in the microflora of the digestive tract cannot be ruled out as factors which might contribute to this decrease in consumption. The difference in nutrient intake would result in a difference in the body weight gains of the treatment groups so that within a very short time the weights of the birds would be different. The difference in body weights would then result in greater differences in feed intake which would cause an even greater disparity in the weight gains. An attempt was made to measure the relative influences of basal diet consumption, average chick weight, ration density and basal diet concentration in the feed on the weight gains of the birds used in the present experiment. Multiple regression equations and correlation coeffi-

TABLE 4
A summary of simple correlation coefficients[1]

Variables		Correlation coefficients	
Y	X	0–42% cellulose[2]	0–42% kaolin
Weight gain	Basal diet consumed	0.969	0.957
	Average chick weight	0.948	0.930
	Ration density	−0.961	−0.911
	Basal diet, gm/ml of feed	0.986	0.945
Basal diet consumed	Average chick weight	0.981	0.943
	Ration density	−0.948	−0.879
	Basal diet, gm/ml of feed	0.959	0.902
Average chick weight	Ration density	−0.974	−0.904
	Basal diet, gm/ml of feed	0.980	0.931
Ration density	Basal diet, gm/ml of feed	−0.997	−0.961
Weight gain	M E Cal./gm of feed	0.967	0.929
	M E Cal./gm of feed	0.976	0.938

[1] Each correlation coefficient had 30 degrees of freedom.
[2] Alphacel.

cients were derived and comparisons were made between the partial regression coefficients as suggested by Snedecor ('56). These analyses proved to be of little value, for, as Snedecor ('56) explains, when a high correlation exists between any two of the X variables "even 6 to 8 significant figures may not be sufficient to control rounding errors." As will be seen from the data of table 4 the variables studied were highly correlated, one with the other.

As the volume of feed which may be consumed appears to be largely regulated by the body weight of the bird to which it is fed, it would seem that the concentration of nutrients within a feed may be of considerable practical importance. A regression analysis between the weight gain (Y) and ME per gram of feed (X) data yielded the highly significant ($P < 0.01$) correlation coefficients 0.967 and 0.929 at 30 d.f. for the diets diluted with cellulose (diets 1–8) and kaolin (diets 1, 9–15), respectively. The regression coefficients 71.20 and 45.84 resulting from this analysis were significantly different ($P < 0.01$). This indicates that the regression equations, while capable of predicting the weight gains of birds once the ME content of 1 gm of feed is known, are limited in use as they differ between types of diets. When the analysis was repeated using the data for weight gain (Y) and for the ME per milliliter of feed (X) the correlation coefficients again proved to be significant ($P < 0.01$): $r = 0.976$ and 0.938 at 30 d.f. for diets 1 to 8 and 1 plus 9 to 15, respectively. The regression coefficients 53.66 and 52.71 were not statistically different suggesting that the data for all diets might be pooled. Analysis of the pooled data yielded the significant ($P < 0.01$) r value of 0.966 at 58 d.f. and the regression equation

$$Y = -119.3 + 52.78X$$

where Y is the gain in weight per bird per week, and X is the ME per milliliter of feed. This demonstrates clearly that the concentration of nutrients in a diet measured in terms of units of nutrient per milliliter is a more useful statistic than concentration measured as units of nutrient per gram of feed. The equation relating to weight gain and ME concentration has been used solely to demonstrate the importance of measuring nutrient density. In the present experiment the ratios of nutrients, one to the other, remained relatively constant as dilution was at the expense of the whole diet; consequently, one would expect that similar results would have been obtained had the concentration of another nutrient been studied.

The influence of nutrient density on weight gain might help to explain such phenomena as the extra-caloric effects of fat (Hill et al., '58) and the improvement of feed utilization by pelleting (Allred et al., '57a, '57b).

SUMMARY

A chick starter diet diluted, by total ration replacement, with zero, 6, 12, 18, 24, 30, 36 and 42% of cellulose and kaolin was fed ad libitum to growing chicks for a three-day ration adustment period followed by a 7-day experimental period. During the latter period, weight gain and feed consumption data were recorded. The 15 diets were each assigned to 4 replicated pens of 14 birds.

The densities of the diets were measured by both a "conventional dry procedure" and by water displacement; the latter proved to be a more satisfactory measure.

The birds receiving the diluted diets attempted to compensate for reduced nutrient concentration by increased feed intake; however, except for the groups receiving diets containing 6% of cellulose and 6 and 12% of kaolin the attempts were unsuccessful. Reduced nutrient intake resulted in decreased weight gains which further limited nutrient intake as this latter variable was largely associated with body weight.

A linear relationship between the concentration of nutrients per unit volume of feed and the weight gains of the chicks suggested that maximum weight gains may be obtained only with diets of high nutrient concentration. Nutrient concentration expressed in terms of nutrients per unit weight did not prove to be as satisfactory a measure as one which took into account ration density.

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Availability of Amino Acids in Maize

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The nutritional quality of a protein depends not only on its amino acid composition and the specific amino acid requirement of the animal but also on the availability of the amino acids.

In vitro digestion studies using proteolytic enzymes have shown that the rate of liberation of the amino acids varied with different types of protein (Melnick et al., '46; Hankes et al., '48; Denton and Elvehjem, '53).

Kuiken and Lyman ('48, '52) were the first to investigate the availability to the rat of amino acids present in proteins by using a modification of Mitchell's "balance sheet" method ('24). Schweigert and Guthneck ('53) determined the availability of lysine in foodstuffs by comparing the weight gain of protein-depleted rats fed graded levels of crystalline lysine, added to a basal ration, with the weight gain of protein-depleted rats receiving a known level of lysine in the foodstuff under investigation. The same workers compared the effect of using normal weanling and protein-depleted rats in an experiment to determine the availability of methionine. They observed that the variation among individual animals was less when weanling rats were used (Schweigert and Guthneck, '54). Gupta and Elvehjem ('57), using weanling rats to determine the availability of tryptophan in several foodstuffs, could not find a significant difference between the values for percentage availability of tryptophan determined by two methods. One involved the determination of unavailable tryptophan in the feces, while the other method made use of the comparison between the growth of rats fed the test ration and the growth of animals receiving rations containing graded levels of tryptophan. These results invalidate somewhat the criticism that bacterial activity in the gastrointestinal tract renders the fecal analysis method unsuitable for availability determination.

Deshpande et al. ('57) using the growth method found that 30% of isoleucine present in zein was available. Except for the observation of Gupta et al. ('58) that lysine, present in maize, was about half available to the rat, no direct studies of the availability of amino acids in maize have been reported.

As part of a study of the effect of fertilizers on the nutritional value of maize, the availability of amino acids was determined. The availability of lysine, isoleucine, methionine and threonine in three maize samples are reported in the present study.

EXPERIMENTAL

Maize samples and experimental rations. The following three maize samples were selected from a fertilizer experiment (De Muelenaere and Quicke, '59):

T_1—maize grown on experimental plots which had not received any fertilizer or manure during 8 years while permanently under maize.

T_2—maize grown on plots which had received an annual application of 4.6 tons of manure per acre.

T_{25}—maize grown on fertilized plots receiving 274 pounds of superphosphate, 91.3 pounds of ammonium sulphate, 91.3 pounds of potassium chloride and 0.9 tons of lime per acre annually.

After harvesting the maize, cobs were allowed to dry for 4 weeks after which they were shelled and dried for another two weeks. The samples were subsequently fumigated with carbon disulphide and stored in large asbestos pots in a refrigerated room. Portions were taken as required for feeding experiments and ground in a hammermill using a 0.024-inch screen.

The composition of the experimental rations is given in table 1.

Experimental procedure. The availability of the amino acids was determined by

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TABLE 1

Composition of experimental rations

Constituents	Low-nitrogen ration	Test rations
	%	%
Defatted whole egg	4.0	—
Corn oil	7.0	4.0
Cellulose powder	2.0	2.0
Salts[1]	5.0	5.0
Choline chloride	0.2	0.2
Inositol	0.1	0.1
Vitamin A and D mixture[1]	0.1	0.1
Vitamin B mixture[1]	1.0	1.0
Cornstarch	80.6	—
Maize	—	87.6

[1] See De Muelenaere and Quicke ('59).

the "balance sheet" method as described by Kuiken and Lyman ('48).

The experiment was carried out with two sets of three pairs of male rats weighing from 70 to 85 gm. Each set was allocated to one of two orthogonal 3 × 3 Latin squares (Fisher and Yates, '57). The advantage of using the two orthogonal Latin squares is that all possible ration sequences have been applied to the animals, which is not the case when only a single square is used. After randomization of columns, position of squares and random allocation of rations to symbols, the final design was as shown in table 2.

Before and after the experiment proper, all animals were fed the nitrogen-low ration (see table 1) in order to measure endogenous amino acid excretion. Each period, including the pre- and post-experimental low-protein periods, had a duration of 8 days, divided into a three-day preliminary and a 5-day collection period. Two per cent of ferric oxide was added before and after each collection period to serve as marker.

The animals were housed individually in galvanized wire cages with raised bottoms and the feces collected on filter paper. The feces of each pair of rats were pooled and dried at the end of the experimental periods. After grinding, aliquots were hydrolyzed for amino acid determinations. The feces of pair 1, period 2, in set 1 were lost accidentally.

The rations were weighed daily and moistened with a small amount of water to reduce spilling. They were offered in clean cups every day and no apparent fermentation took place during the 24-hour period. The amount of food consumed was determined with suitable corrections for the water added.

The amino acids were determined in the rations and the feces by microbiological assay using *Leuconostoc mesenteroides* for methionine, isoleucine and lysine, and *Streptococcus faecalis* for threonine determination (Barton-Wright, '52).

RESULTS AND DISCUSSION

The results of the availability determinations for lysine are shown in table 3. The treatment means expressed as percentage availability of lysine are 90.4, 89.3 and 88.9 for A, B and C, respectively. No significant differences were found among the values for the availability of lysine for the respective maize samples. The fact that the availability of lysine in the present study is considerably higher than the values reported by Gupta et al. ('58) may possibly be attributed to the difference in assay method used in the respective studies.

The growth method may be subject to several errors inherent in the method, particularly when poor quality proteins such as cereal proteins are tested for amino acid availability. The possibility of an increased lysine requirement as the result of an increase in the protein level of the

TABLE 2

Layout of the cross-over design[1]

Period	Set 1			Set 2		
	Pair 1	Pair 2	Pair 3	Pair 1	Pair 2	Pair 3
1	A	B	C	A	B	C
2	B	C	A	C	A	B
3	C	A	B	B	C	A

[1] Symbols: A = T_{25}, B = T_1, C = $T_{\frac{1}{2}}$.

TABLE 3
Availability values of amino acids in maize

	Set 1								
Period	1			2			3		
Pair no.	1	2	3	1	2	3	1	2	3
Ration	A	B	C	B	C	A	C	A	B
Lysine availability	90.9	93.9	91.8	—	84.3	89.8	90.8	88.3	89.1
Isoleucine availability	93.4	93.1	92.6	—	86.6	96.9	91.2	84.3	91.8
Threonine availability	95.3	93.4	92.1	—	91.1	95.7	79.5	72.5	85.2
Methionine availability	94.8	95.2	96.0	—	92.6	95.5	95.7	92.7	95.5

	Set 2								
Period	1			2			3		
Pair no.	1	2	3	1	2	3	1	2	3
Ration	A	B	C	C	A	B	B	C	A
Lysine availability	93.6	89.7	86.4	90.2	90.3	91.5	85.1	89.9	89.7
Isoleucine availability	95.1	92.7	91.8	94.9	96.8	97.4	88.4	95.4	93.9
Threonine availability	92.7	90.5	89.5	96.0	88.7	87.2	83.6	85.9	83.0
Methionine availability	96.6	95.7	95.9	94.4	95.4	96.5	94.6	96.2	96.8

ration by the addition of 50% of maize must be considered. Evidence that the requirement for the most limiting amino acid in a diet increases with a rise in protein level was reported as early as 1948 by Grau. From the work of Munaver and Harper ('59) it is evident that an increase of 6% of wheat gluten in a ration, supplemented with lysine to contain a total of 0.9% of the latter, depressed growth of rats by approximately 17 gm during a period of two weeks. Since 50% of maize increases the protein content of the ration by roughly 5%, the requirement for lysine may have risen significantly to result in a lower value for availability of lysine as compared with the availability of lysine in the better quality proteins which were added to the test ration at a lower level.

The fact that 50% of maize raises the total fat content of the test ration by about 2% may result in a further increase in lysine requirement (Rosenberg and Culick, '55) and thus lower the relative availability of lysine.

As with lysine no significant difference between the availabilities of isoleucine can be established. The respective means for A, B and C are 93.4, 92.9 and 92.1. Although no direct study on the availability of isoleucine in maize has been published, the values reported presently are high when compared with the availability of isoleucine in zein. Since about 50% of the isoleucine present in maize is situated in the zein fraction and only 30% of the isoleucine in zein is available (Deshpande et al., '57), as established by the growth method, a lower availability of this amino acid in maize would be expected.

In addition to the criticism offered earlier for the determination of availability of lysine by the growth method, a further error may yet be introduced by the complex interrelationship between leucine, isoleucine and valine. The low valine content in zein, which was recently found to be interrelated with the leucine-isoleucine antagonism (Benton et al., '56) warrants particular attention in an experiment in which growth is used as the criterion for availability. However the fact that Deshpande et al. ('57) were able to show that the availability of isoleucine in the acid hydrolysate of zein was twice that of the zein, without question points to an impaired availability of this amino acid in the protein. On the basis of this and in view of the high availability of isoleucine in maize, as found in the present study, the question may be raised whether the availability of amino acids present in zein has not been impaired by the isolation process. This aspect needs further investigation.

The availability values for threonine with means of 88.0, 89.4 and 89.0 for sample A, B and C respectively, does not reveal any significant difference between the samples. The availabilities in period 3

for both set 1 and set 2 are significantly lower than for any of the other periods. No explanation can be offered for this.

The availability means for methionine are 95.3, 95.4 and 95.1, respectively, for samples A, B and C. No significant differences were found among the three samples.

The average endogenous amino acid excretion as percentage of the total amino acid excreted by animals fed the maize ration was found to be 72, 77, 60 and 64% for lysine, isoleucine, threonine and methionine, respectively.

It is clear from the above experiments that the different fertilizer treatments do not have a significant influence on the availability of the individual amino acids present in the maize.

It is felt by the authors that the method used to determine availability in this study, although sometimes criticized because of changes possibly brought about by microorganism activity in the intestinal tract, is the only safe method for availability determinations of amino acids in poorly balanced proteins.

Since no imbalances or antagonisms are likely to be created by the addition of good quality protein to the basal ration, the growth method may be more justifiable for them. This is borne out by the agreement between availabilities obtained from growth and fecal analysis (Gupta and Elvehjem, '57). When, however, zein and other poorly balanced protein sources are added to a basal ration growth rates are not the sole reflection of the availability of the particular amino acid assessed but also become dependent further on the composition of the protein. The use of amino acid mixtures in the basal ration may eventually be able to correct these imbalances and antagonisms which arise from the addition of poorly balanced proteins, but more information is necessary in this field before this method can be applied successfully.

SUMMARY

The availability of lysine, isoleucine, threonine and methionine was determined in three samples of maize grown under different fertilizer conditions. No significant differences in availability were found among the three samples for the respective amino acids. Methods for the determination of biological availability of amino acids are discussed briefly.

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Nonsynthesis of Linoleic Acid from Acetate-1-C^{14} by the Laying Hen[1]

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In previous studies in this laboratory on the deposition of fatty acids in eggs, it was concluded from balance experiments that the hen is able to synthesize dienoic acid *de novo* and deposit it in the egg (Reiser, '50, '51). However, Bernhard and Schoenheimer ('40) found that rats, which incorporated deuterium from body water into other fatty acids, incorporated none into linoleic acid isolated as the tetrabromide. Mead and associates ('53) also injected rats intraperitoneally with Carbon-14 labeled acetate and found that linoleic acid, isolated from their depot fat as the tetrabromide, had little activity.

Since the isotope labeling technique is more accurate than the earlier balance studies of Reiser ('50, '51), the present study was designed to determine whether the hen can incorporate labeled acetate into linoleic acid.

EXPERIMENTAL

A white leghorn hen in full production was fed a low-fat diet for 6 weeks. The composition of the diet is presented in table 1.

TABLE 1

Composition of the low-fat diet[1]

	%
Glucose[2]	68.66
Soybean protein[3]	20.00
Minerals[4]	9.84
Vitamins[4]	1.50

[1] 100 ml of 40% choline chloride were added to 100 pounds of the diet.
[2] Cerelose, Corn Products Refining Company, New York.
[3] Drackett Assay Protein C-1, Drackett Company, Cincinnati.
[4] For composition, see Wheeler et al. ('60).

The hen was then injected intraperitoneally with 0.33 μc of sodium acetate-1-C^{14}. Three, 15 and 16 days afterward injections were made of 0.66, 0.50 and 0.50 μc, respectively. The eggs laid subsequently were collected. Fourteen eggs were analyzed separately. Seven of the 14, the ones having the highest specific activity, were used for the analysis. Each yolk was mixed with 200 ml of chloroform in a Waring blender for 10 minutes. The whole mass was centrifuged, and the chloroform extract of the total egg lipid was shaken with 50 gm of silicic acid for 10 minutes and then filtered through a sintered glass funnel, using reduced pressure. The silicic acid was washed with 100 ml of chloroform (Van Handel and Zilversmit, '57). The chloroform washings from each yolk, containing the triglyceride and sterol fractions, were pooled, reduced in volume and stored at − 20°C. The chloroform-washed silicic acid was shaken with 100 ml of absolute methanol and filtered through a sintered glass funnel. After repeating the washing three times, the methanol extracts, containing the phospholipid fraction, were pooled, reduced in volume and stored at − 20°C. The radioactivities of the triglyceride plus sterol fraction and the phospholipid fraction were determined and the lipids from the more active eggs were fractionated. The fractionation scheme for egg fatty acids and their relative specific activities are shown in figure 1.

The phospholipid fractions and the triglyceride plus sterol fractions were each pooled, saponified with 4% alcoholic KOH, the soaps decomposed with 2 N H_2SO_4, and the fatty acids extracted with 30 to 60° petroleum ether. The specific activity of the mixed triglyceride fatty acids ([1], fig. 1) was 1552 cpm/mg, and that of the phospholipid fatty acids ([2], fig. 1) was 1589 cpm/mg.

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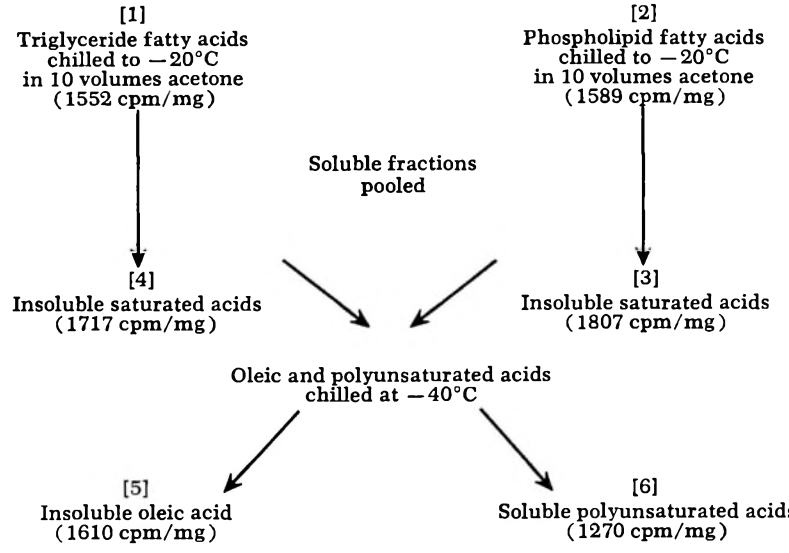

Fig. 1 The fractionation scheme for egg fatty acids.

The fractions 1 and 2 were dissolved in 10 volumes of acetone and subjected to low temperature crystallization according to the method of Brown ('41). The acetone solutions were chilled at − 20°C overnight. The insoluble fractions (saturated acids) were removed by filtration through a chilled sintered glass funnel. The soluble fractions from [1] and [2] (oleic, polyunsaturated and traces of saturated acids) were pooled and chilled again at − 20°C.

The − 20°C filtrate was held at − 40°C (acetone-dry ice bath) for three hours. The soluble fraction was removed by vacuum filtration. The filtrate was somewhat reduced in volume and again held at − 40°C for more complete removal of oleic acid from the polyunsaturated fraction. The fractions obtained by low temperature crystallization were counted and their specific activities determined on "frosted" glass planchets[2] in a gas flow counter with a micro-mil window.

Fractions 1 to 6 were analyzed for their fatty acid composition by the use of a thermal conductivity detector at 199°C and a flow rate of 70 ml/min. of helium.

The linoleic acid entrapped in a 500-mg sample of fraction 5 was recovered as the tetrahydroxy derivative and its specific activity determined as follows. The sample was oxidized with potassium permanganate by the method of Sullivan and Bailey ('36). The fatty acids were converted into their sodium salts and dissolved in 100 ml of water. After cooling in an ice bath, 50 ml of 1% potassium permanganate were added with continuous stirring at lower than 10°C. After allowing the reaction to continue for another hour, the excess of permanganate was decomposed with sodium bisulphite and hydrochloric acid. The mixture was allowed to stand for 24 hours, after which the precipitate was filtered. The precipitate was washed with petroleum ether to remove all the saturated and unoxidized acids. The washed precipitate was then continuously extracted with ether for 20 hours in a Soxhlet extractor. The ether extract was evaporated and the dihydroxystearic acid recrystallized from ethyl acetate. The residue remaining after ether extraction was recrystallized from ethanol and assumed to be tetrahydroxystearic acid.

One microliter of methyl esters of fraction 6 was separated by gas chromatography into 4 fractions: (a) methyl esters of C_{14} and C_{16} acids, (b) methyl oleate, (c) methyl linoleate and (d) methyl esters of polyunsaturated acids. An ionization detector was used. The methyl esters were

[2] PL-F Full-Blasted Planchets, B and Z Enterprizes, Columbus, Ohio.

TABLE 2
Fatty acid composition of the fractions[1] as determined by gas liquid chromatography

Acids	1	2	3	4	5	6
	%	%	%	%	%	%
14:0[2]	0.50	0.48	0.50	0.54	0.32	1.12
14:1	0.20	0.22	0.18	0.20	0.21	0.51
16:0	36.80	26.03	63.77	53.46	20.45	2.36
16:1	6.87	5.08	0.90	3.95	3.95	17.28
16:2	0.00	0.00	0.47	0.00	0.00	0.00
18:0	9.66	10.41	27.56	9.81	2.84	0.00
18:1	42.85	51.92	5.77	31.02	71.39	69.26
18:2	2.33	2.05	0.37	0.76	0.59	6.18
20:0	0.00	0.11	0.09	0.00	0.00	0.06
18:3	0.72	0.64	0.09	0.26	0.25	1.08
18:4	0.00	0.87	0.08	0.00	0.00	0.67
20:3 or 22:0	0.00	0.34	0.00	0.00	0.00	0.00
20:4	0.00	1.85	0.21	0.00	0.00	1.48

[1] 1. Triglyceride fatty acid.
2. Phospholipid fatty acid.
3. Phospholipid fatty acid insoluble at −20°C.
4. Triglyceride fatty acid insoluble at −20°C.
5. Pooled triglyceride and phospholipid fatty acid soluble at −20°C, but insoluble at −40°C.
6. Pooled triglyceride and phospholipid fatty acid soluble at −40°C.
[2] Number of carbons:number of double bonds.

made by the action of diazomethane on the free fatty acids. The liquid phase was 20% ethylene-glycol-succinate on chromosorb W (60 to 80 mesh) in a 6′ by ¼″ column. The column temperature was 195°C and the flow rate 60 ml of argon gas/min. All the fractions except the linoleate were collected directly into a liquid scintillator (4 gm of 2,5-diphenyloxazole, and 100 mg of 1,4-bis-2-[phenyloxazolyl] benzene/l of toluene). Methyl linoleate was collected in toluene and half the total solution was assayed for purity by gas chromatography. The solvent was removed from the other half of the solution and the liquid scintillator added.

RESULTS AND DISCUSSION

The fatty acid compositions of fractions 1 to 6 as determined by gas chromatography are given in table 2. The radioactivity of the fraction obtained by permanganate oxidation were also counted on the glass planchets and are presented in table 3.

Since the efficiency of the counter was about 25%, the activity of the linoleic acid was approximately 25 dis./min./mg. From table 3 it may be seen that the linoleic acid has little or no activity, the low level recorded being easily accounted for by contamination and experimental error.

TABLE 3
Radioactivity of the fractions obtained from the permanganate oxidation

Fraction	cpm/mg
Saturated acids	1736
Oleic acid[1]	1149
Linoleic acid[2]	6

[1] Isolated as dihydroxystearic acid.
[2] Isolated as tetrahydroxystearic acid.

TABLE 4
Radioactivity of fatty acid fractions (from fraction 6) obtained by gas chromatography

Fraction		cpm/mg
(a)	C_{14} and C_{16} methyl esters	2178
(b)	Methyl oleate	2036
(c)	Methyl linoleate	13
(d)	Higher polyenoic esters	1887

Linoleic acid (from fraction 6) was also isolated by gas liquid chromatography. The fractions collected in the liquid scintillator were counted in a Tri-Carb liquid spectrometer.[3] The counts obtained are shown in table 4.

[3] Packard Instrument Company, Inc., La Grange, Illinois.

The efficiency of the gas flow counter was 25%. Therefore the actual activity of linoleic acid isolated as the tetrahydroxy derivative, and presented in table 2, was about 25 dis./min./mg. Since the efficiency of the liquid spectrometer was 45%, the activity of gas chromatographically isolated linoleic acid was about 30 dis./min./mg. Considering the errors of the methods, this value is the same as that obtained by the isolation of the tetrahydroxy derivative, and may be attributed to contamination and experimental error.

CONCLUSIONS

The laying hen does not synthesize linoleic acid *de novo*. Any of this important nutrient which occurs in the egg must first be in the diet. This is contrary to the conclusion of previous studies based on the presence of small amounts of linoleic acid in hens' eggs after hens received a fat-free diet for one year.

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Effect of Feeding Vitamin K-Deficient Diets to Female Rats[1]

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Occurrence of a hemorrhagic syndrome in the growing male rat fed irradiated beef, apparently caused by vitamin K deficiency, has been reported by Metta et al. ('59). Ever since the first observation of this syndrome in the authors' laboratories in 1956, it has been evident that the female rat is not as susceptible to vitamin K deficiency as the male. Several hypotheses have been suggested to explain this resistance of the female rat to vitamin K deficiency. One is that the female may practice coprophagy to a greater extent than the male, and thus may obtain more vitamin K from the feces. Mameesh et al. ('59) and Barnes et al. ('59) have shown that coprophagy is an important means of the rat's obtaining vitamin K. A second hypothesis is that the male, because of his greater food intake and greater body weight gain may develop a vitamin deficiency more rapidly than the female, on the basis that the more of a deficient diet an animal consumes, the faster he will become deficient. A third possibility is that there is an actual sex difference under hormonal control.

Animal experiments were undertaken to determine whether the greater resistance of the female rat to vitamin K deficiency was due to a greater practice of coprophagy, to the lower food intake and slower rate of growth of the female or to a sex difference. In addition, the effect of feeding a vitamin K-free diet on reproduction of the female was also studied. The results indicate, for the first time, that there is a difference in vitamin K requirement due primarily to sex and that the female rat needs very little dietary vitamin K for growth and reproduction, even when coprophagy is prevented.

EXPERIMENTAL AND RESULTS

Resistance of the female rat to vitamin K deficiency. Experiment 1. During a 13-month longevity-reproduction study, male and female Sprague-Dawley rats were fed ad libitum an irradiated beef diet of the following composition per 100 gm of diet (in grams): ground beef (γ-irradiated at 2.8 million rad) 36; cornstarch, 35; sucrose, 19; glucose,[2] 5; cod liver oil, 1.5; wheat germ oil, 0.5; mineral mix 446 (Mameesh and Johnson, '58), 4; choline chloride, 0.1; and adequate amounts of the B-vitamins (Metta et al., '59). During these tests, hemorrhagic deaths occurred in 78 of 138 growing male rats (the first death was observed on the 9th day), but in only two of 90 growing female rats (deaths occurred on the 188th and 364th day). On the other hand, when a similar diet containing non-irradiated beef was fed to 40 male and 40 female rats, three males died from hemorrhage (on the 105th, 131st and 300th day, respectively), whereas no females died.

Experiment 2. To study the effect of food intake, rate of gain and coprophagy on susceptibility to vitamin K deficiency, 30 male and 25 female rats were divided into 5 groups and fed the irradiated beef diet according to the experimental plan given in table 1. Using this design, we were able to control two variables, namely, food intake and body weight gains, and to study the effect of coprophagy in the female. The male rats in group 4, which were fed the irradiated beef diet ad libitum, served as controls. Rats in groups 1 through 4 were housed in cages with raised screen

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[2] Cerelose, Corn Products Refining Company, New York.

TABLE 1

The comparative effect of feeding an irradiated beef diet to growing male and female rats

Group	Sex	Method of feeding	Coprophagy allowed or prevented	No. of hemorrhagic deaths	Mean plasma prothrombin time after 56 days
				%	*seconds*
Experiment 2					
1[1]	Male	restricted; feed intake equated with that of group 2	allowed	80	36(28–44)[3]
2[1]	Female	ad libitum	allowed	0	13(12–16)
3[1]	Male	restricted feeding; body gains equated with that of group 2	allowed	50	18(16–21)
4[1]	Male	ad libitum	allowed	100	—
5[2]	Female	ad libitum	prevented	0	17(14–19)

[1] Ten rats per group.
[2] Group 5, 15 rats.
[3] Range of values.

bottoms; thus, all these rats had access to their feces. The female rats of group 5 were housed in coprophagy-prevention tubular cages (described elsewhere in the text) and fed ad libitum.

As can be seen in table 1, the male animals in all groups were very susceptible to vitamin K deficiency, whereas not a single female rat had succumbed from vitamin K deficiency after eating the irradiated beef for as long a period as 56 days. After 36 days of feeding, 5 coprophagy-prevented female rats were killed and plasma prothrombin times were measured (Quick, '38). The individual values were 14, 16, 20, 15 and 15 seconds, which are in the normal range. After 56 days, plasma prothrombin times were determined on the surviving females (except 3 females from group 5 which were held for reproduction study); these were again in the normal range, namely, 15–19 seconds for the coprophagy-prevented rats, and 11–15 seconds for the coprophagy-allowed rats.

Reproduction in the irradiated beef-fed female rats. Experiment 3. Three female rats from group 5, which had been housed in tubular cages to prevent coprophagy while being fed the low-vitamin K irradiated beef diet, were continued on experiment for 80 days. At the end of this time they were transferred to regular screen-bottom cages and mated with normal male rats. They conceived and gave birth to 11, 10 and 8 pups, respectively. Although 15 of these pups died within 15 days, evidence of hemorrhage was not observed in them. The fact that when male and female rats were fed the same diet, essentially normal reproduction-gestation occurred in the female rats and all males died of vitamin K deficiency, further emphasizes the marked resistance of the female to this vitamin deficiency.

Synthetic diets and vitamin K nutrition of the female rat. Experiment 4. Since the feeding of the irradiated beef diet resulted in vitamin K deficiency in the male rat, but only rarely in the female rat even when coprophagy was prevented, further studies were carried out using vitamin K-free synthetic diets which had previously been found to cause vitamin K deficiency in the male (Mameesh et al., '59).

Five male and 4 female weanling rats of the Sprague-Dawley strain were housed in screen-bottom cages and fed ad libitum a synthetic diet of the following composition per 100 gm of diet: casein,[3] 25 gm; DL-methionine, 0.5 gm; sucrose, 54 gm; glucose, 5 gm; corn oil,[4] 5 gm; lard, 1.5 gm; glycerine, 3 gm; mineral mix 446 (Mameesh and Johnson, '58), 5 gm; cellu-

[3] Nutritional Biochemicals Corporation, Cleveland.
[4] Mazola, Corn Products Refining Company, New York.

TABLE 2

Effect of feeding vitamin K-free synthetic diet to male and female growing rats

Group	No. of rats	Sex	Days on experiment	Coprophagy allowed or prevented	No. of hemorrhagic deaths	Mean plasma prothrombin time at end of experiment
						seconds
			Vitamin K-free synthetic diet			
Experiment 4						
1	5	Male	16	allowed	0	29(25–33)[1]
2	4	Female	44	allowed	0	17(15–19)
Experiment 5						
3	4	Male	14	prevented	2[2]	74(44–98)
4	7	Female	94	prevented	2[3]	—
			Vitamin K-free synthetic diet plus sulfathalidine			
5	7	Male	26	allowed	2[4]	47(21–94)
6	7	Female	26	allowed	0	34(15–96)

[1] Range of values.
[2] Two male rats died from hemorrhage on the 12th day of feeding.
[3] Two female rats died from hemorrhage on the 62nd and 69th day, respectively.
[4] Two male rats died from hemorrhage on the 24th day.

lose,[5] 1 gm; vitamin A,[6] 1000 I. U.; vitamin D, 100 I.U.; α-tocoperyl acetate,[7] 15 mg; choline chloride, 100 mg; Ca-D-pantothenate, 5 mg; nicotinic acid, 2 mg; thiamine·HCl and riboflavin, 1 mg each; pyridoxine·HCl, 0.5 mg; folic acid, 100 μg; vitamin B_{12}, 5 μg; and biotin, 3 μg.

Data in table 2 summarize the results of experiment 4. On the 16th day of feeding, male rats were taken off the experiment and plasma prothrombin levels were determined; a mean value of 29 seconds was obtained, showing that hypoprothrombinemia had been produced in the male rats fed the vitamin K-free diet in 16 days, despite coprophagy. The female rats were continued on the regimen for a total of 44 days, at the end of which time the mean plasma prothrombin value was found to be 17 seconds, a normal prothrombin level even after this long period on the vitamin K-free regimen. However, since there was a possibility that the female rat had obtained a larger vitamin K supply by coprophagy, the following experiment was carried out.

Experiment 5. Since effective coprophagy prevention in the female rat is difficult using the tail-cup method (Barnes et al., '59), square-tubular cages were made from half-inch-square hardware cloth. A 4-inch overhang allowed room to screw in a feed jar, and a square piece of metal, to serve as a door, was inserted near the end of the overhang. The size of the tube permitted the rat fairly comfortable living, but not sufficient room to turn and gain access to its feces.

The vitamin K-free synthetic diet was fed ad libitum to weanling Sprague-Dawley rats, two groups being fed in the tubular cages and two in regular cages. The same basal diet, except for 1% of sulfathalidine replacing sucrose in order to reduce bacterial synthesis of vitamin K, was fed to two additional groups housed in regular cages. Inclusion of sulfathalidine in the diet resulted in hemorrhagic deaths of the male rats, but not of the females. The design of the experiment and the results are given in table 2.

After 94 days of housing in tubular cages, receiving a vitamin K-free synthetic diet, 5 female rats were transferred to regular cages and mated with control male rats which had been raised with laboratory chow,[8] to study again the effect of dietary vitamin K deficiency on reproduction. All 5 females conceived and gave birth to live pups. However, although most

[5] Woodflock, Brown Company, Chicago.
[6] Myvax, a concentrate of vitamin A palmitate and activated ergosterol, sold by the Distillation Products Industries, Rochester, New York.
[7] α-Tochopheryl acetate, choline chloride and most of the B-vitamins were generously supplied by Merck and Company, Rahway, New Jersey.
[8] Purina Chow, Ralston Purina Company, St. Louis.

of the pups died within 5 days, none showed hemorrhagic symptoms.

In our earlier studies on the longevity and reproduction performance of female rats maintained with irradiated beef diets (Metta et al., '59) supplemented with 0.1 μg of vitmain K_3 (menadione) per gm of dry diet, practically no pups survived following the first mating and about 35% survived following the second mating with stock male rats. However, when the rats were maintained in an isolation room free from respiratory disease, 66 to 74% survival of the pups was obtained.

The very great variation in susceptibility to vitamin K deficiency due to difference in sex, found in the first experiment, although more appreciable, is in the same direction as that reported by Nightingale et al. ('47) for vitamin K deficiency induced by dihydroxy-stearic acid. They found that hypoprothrombinemia developed in 30 to 42 days in the male, and in 56 to 63 days in the female rats, fed the dihydroxystearic acid diet.

In the second experiment 100% of the male rats fed the irradiated beef diet ad libitum, and 80% of the male rats fed an amount of diet equal to that of the female rats fed ad libitum, died from hemorrhage, whereas the female rats showed no symptoms of the deficiency. Furthermore, when male rats were fed amounts of the irradiated beef diet which caused the same rate of growth as that of the ad libitum-fed female rats, 50% of the males died from hemorrhage. This observation suggests that the greater susceptibility of the male to vitamin K deficiency is not due to a greater food intake or greater rate of gain by the male; and the normal prothrombin times obtained regardless of whether the female rats were or were not allowed to practice coprophagy indicate that the resistance of the female rat to vitamin K deficiency is not due to increased vitamin K content of the feces or increased fecal consumption. Even a vitamin K-free diet containing sulfathalidine was not as harmful to female rats as it was to male rats.

The data suggest that the difference in susceptibility of male and female rats to vitamin K deficiency is due strictly to sex difference and that the female sex hormones are probably related in some way to vitamin K requirement and function. Further support for this hypothesis is given in Mellette's report ('60) that castration or administration of stilbestrol (but not testosterone) prevented male rats from succumbing to vitamin K deficiency when fed irradiated beef diets.

SUMMARY

The female rat was very much less susceptible to vitamin K deficiency than the male. This increased resistance of the female rat to vitamin K deficiency was not affected by the prevention of coprophagy, nor was it due to lower food intake or slower rate of growth, but was apparently the result of a true sex difference presumably under hormonal control.

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Requirement and Utilization of Iron by the Baby Pig[1,2]

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Assessment of the dietary iron requirement of an animal directly implicates its utilization since iron absorption is a function of body need (Hahn et al., '39, '43). Iron need, presumably, is largely a function of (a) rate of growth and (b) turnover of the red blood cells. The basic parameter for growth is the amount of iron in hemoglobin formed *de novo*, and that for maintenance, the rate of destruction of red blood cells and the degree of reutilization of red cell iron. The consequences of these assumptions are that, iron utilization being equal, a fast-growing animal would require more iron than a slow-growing animal, and in addition, that the iron need for growth would dominate that of maintenance (Matrone et al., '57).

With these factors in mind the present study was initiated to investigate the utilization and minimum iron requirement of the pig during the period of its most rapid rate of growth.

EXPERIMENTAL

The experimental subjects in the three experiments presented were 38 newborn pigs deprived of colostrum. At birth 10 ml of porcine gamma globulin[3] were administered to the piglets, 5 ml intraperitoneally and 5 ml subcutaneously (Barrick et al., '54). They were put into individual aluminum cages, and fed from pyrex dishes. The basal diet was fresh cows' milk fortified with vitamins A and D, 15 ppm of manganese[4] and 30 ppm of zinc, moisture-free basis. Each pig was allowed all that it would consume in each of 5 daily feedings. Under this dietary regimen the milk intake per pig ranged from 120 to 220 ml/kg of body weight/day.

In the first experiment 12 baby pigs were fed the basal diet, which contained 1.8 ppm of iron, moisture-free basis, until they became anemic (approximately 6 gm of hemoglobin per 100 ml blood). They were stratified in three groups of 4 according to weight and hemoglobin level and allotted to the following diets in a randomized block design: (1) basal + 5 ppm Cu + 10 ppm of Fe; (2) basal + 5 ppm of Cu + 20 ppm of Fe; (3) basal + 5 ppm Cu + 40 ppm of Fe; and (4) basal + 5 ppm of Cu + 80 ppm of Fe (ppm expressed as dry matter of diet). The additions of iron were above the 1.8 ppm present in the milk.

The same 4 diets or levels of supplemental iron were studied in experiments 2 and 3 except that the piglets were placed on experiment immediately after birth without undergoing depletion. In the third experiment a 5th diet or level of Fe, consisting of the basal + 5 ppm of Cu + 60 ppm of Fe, was added. Iron and copper analyses of the milk diets were made biweekly.

In experiments 1 and 2, each piglet received one dose of 5 μc of Fe^{59} intraperitoneally at the beginning of the experimental period. At 5-day intervals body weights were recorded and blood samples for radioactivity and hemoglobin determinations were taken from ear veins. At the

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[2] Supported in part by grants from the Herman Frasch Foundation and Moorman Manufacturing Company, Quincy, Illinois.

[3] Porcine gamma globulin was provided by the Armour Laboratories through the courtesy of Dr. M. A. Schooley.

[4] Supplemental metals supplied as reagent grade salts of $MnSO_4 \cdot H_2O$; $FeSO_4 \cdot 7\,H_2O$; $CuSO_4 \cdot 5\,H_2O$ and ZnO.

termination of each experiment the pigs were exsanguinated by severing the jugular vein. The liver, heart, spleen and kidneys were excised, weighed, and radioactivity was determined. In addition, radioactivity of a sample of bone marrow from the right femur and of muscle from the right ham was also measured. A similar procedure was followed in experiment 3 except that those steps associated with radioactivity measurements were omitted.

Hemoglobin was estimated by the method of Shenk et al. ('34). Blood samples were prepared for beta counting in a proportional counter[5] by diluting 0.02 to 5 ml with 0.1% Na_2CO_3 in a hemoglobin pipette. The solution was transferred to a tared aluminum planchet over a circular area of constant diameter and dried. A self-absorption curve of radioactive Fe^{59} blood was also prepared for correcting sample counts to infinite thinness. Samples of the tissues weighing between 4 and 5 gm were wet-ashed with nitric acid and gamma activity was determined with a well scintillation counter.[6] Radioactivity of Fe^{59} standards, prepared for beta and gamma counting, was measured each time samples were counted in order to make corrections for radioactive decay. The method outlined by Parks et al. ('43) as modified by Matrone et al. ('47) was used for the determination of iron.

RESULTS AND DISCUSSION

Requirements

Changes in hemoglobin. The data of the hemoglobin changes observed in experiment 1 are shown in figure 1. One pig receiving the 20 ppm and one receiving 40 ppm of Fe died after 15 days on experiment of causes presumed to be other than those attributable to the experimental treatments. Thus, the curves for these levels of iron shown in figure 1 are averages from two pigs instead of three. The hemoglobin values of the pigs receiving the 10 ppm level of Fe continued to drop precipitously so that at the end of 30 days the average was less than 3 gm per 100 ml. At the end of 35 days all three pigs receiving the 10 ppm of Fe had died in an extremely anemic condition. Their blood at termination was straw colored, and autopsy revealed yellow livers and enlarged hearts. It is apparent from the graph that 20 ppm of Fe was also inadequate; the 40 ppm level of Fe approached sufficiency, and the 80 ppm level of Fe appeared to be adequate.

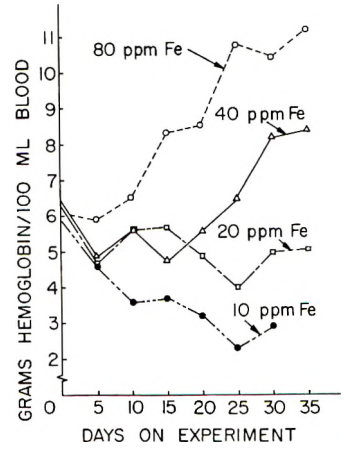

Fig. 1 Effect of dietary levels of iron in parts per million of dry matter on hemoglobin level of baby pigs made anemic prior to initiation of iron feeding.

The hemoglobin data of experiment 2 are shown in figure 2. The curve for the 10 ppm of Fe is the average from two pigs since one pig receiving 10 ppm of Fe died after 30 days on experiment in a severe anemic state but the other two survived the 60-day period of the experiment. It is apparent from figure 2 that the hematopoietic responses were similar to those in experiment 1, even though the pigs in experiment 2 were not depleted of iron previous to the initiation of the experimental period.

Since the results obtained in the first two experiments indicated that the minimum iron requirement was between 40 and 80 ppm of Fe, a level of 60 ppm was added to the previously used levels in experiment 3. The changes in hemoglobin values are shown in figure 2. Only two pigs were allotted to the 10 ppm level of iron in this experiment, and one pig, receiving the 20 ppm of Fe, died after 10 days on experiment. Therefore, the values plotted in figure 2 for the 10 and 20 ppm

[5] Nuclear Measurements Corporation, Indianapolis 18, Indiana.

[6] Nuclear Chicago Corporation, Chicago 10, Illinois.

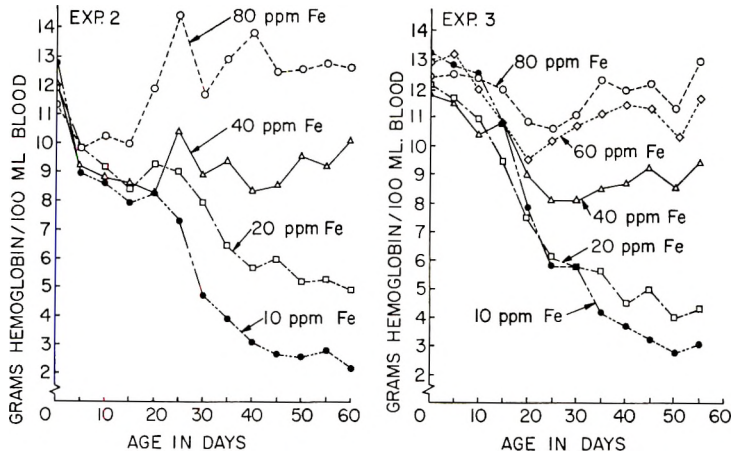

Fig. 2 Experiment 2, effect of 4 levels of iron on hemoglobin levels of baby pigs. Experiment 3, effect of 5 levels of iron on hemoglobin levels of baby pigs.

of Fe are averages from two pigs, whereas the values plotted for the other levels of iron are averages of three pigs. Again it is apparent from the graph that the 10 and 20 ppm of Fe and quite probably the 40 ppm are inadequate whereas either the 60 or 80 ppm of Fe appears to be sufficient. Thus, evaluation of the hemoglobin data of these three experiments suggests that the minimum dietary iron requirement of the baby pig is approximately 60 ppm.

Body and tissue weight results. The weight gains obtained in the three experiments are summarized in table 1. Animals receiving the 10 ppm of Fe gained significantly less ($P \leq 0.01$) than pigs receiving other levels of iron, in all three experiments. In experiments 2 and 3 pigs receiving the 20 ppm level of iron gained less ($P \leq 0.05$) than those receiving the 40 and 80 ppm of Fe. The differences in gains among pigs receiving either the 40, 60 or

TABLE 1

Effect of level of dietary iron on weight gains of baby pigs

Fe level	No. of animals	Initial weight	35-Day gain	Final gain
ppm		*gm*	*gm*	*gm*
		Experiment 1 (30 days)		
10	3[1]	2902	—	5,430
20	2[2]	2887	—	9,749
40	2[2]	2733	—	10,124
80	3	2802	—	11,178
		Experiment 2 (60 days)		
10	2[2]	975	3880	7,304
20	3	1083	3367	13,177
40	3	1072	3475	16,747
80	3	1008	3549	17,360
		Experiment 3 (55 days)		
10	2	1383	5995	10,211
20	2[2]	1335	6497	14,347
40	3	1197	6672	17,879
60	3	1300	6948	19,735
80	3	1307	6562	19,275

[1] All three pigs died before the end of 35-day period (see text).
[2] One pig died during course of experiment (see text).

80 ppm levels of iron were not significantly different ($P \geq 0.05$). These results suggest that weight gains are somewhat less sensitive to iron status than is hemoglobin level. It is interesting to note, table 1, that gains of all pigs in experiments 2 and 3 were quite similar until the 35th day of the experiment. Thereafter, differences between the lower and higher levels of iron developed and by the end of the experiment were readily apparent. The data in figures 2 and 3 suggest that the critical hemoglobin level in respect to growth is about 5 gm.

The effect of level of dietary iron on heart and liver weights is shown in table 2. The average heart weight expressed in grams per kilogram of body weight was two to three times greater for the pigs fed the 10 ppm level than for those fed the higher levels of iron; this difference was highly significant ($P \leq 0.01$). A significant difference for this contrast was also obtained in a covariance analysis with heart weight and body weight as covariables. Thus, the differences in heart weights were not attributable to differences in body weight. No evidence was obtained from these statistical analyses that the heart weights of the pigs receiving the 20, 40 or 80 ppm levels of iron were significantly different from each other. These data suggest that heart weight also is less sensitive to iron deficiency than is hemoglobin level. Presumably, the enlargement of the heart is a consequence of the compensatory action of this organ in response to the stress brought about by the low oxygen carrying capacity of blood prevailing in severe iron anemia.

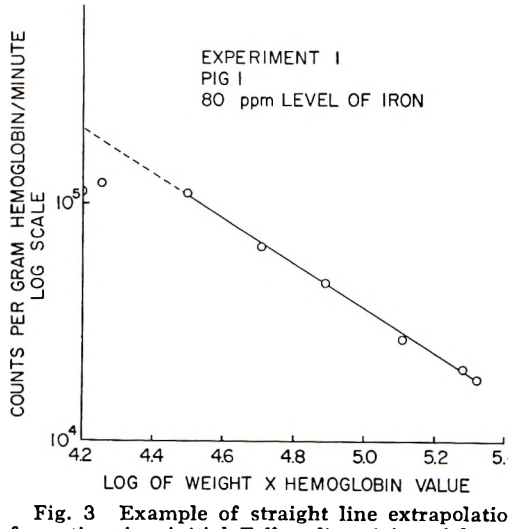

Fig. 3 Example of straight line extrapolation for estimating initial Fe^{59} radioactivity of hemoglobin free of incorporation time.

TABLE 2

Effect of level of dietary iron on heart and liver weight per kilogram of body weight of pigs in experiments 1 and 2[1]

Fe level	Heart weight/ kg body weight	Liver weight/ kg body weight
ppm	*gm*	*gm*
10	13.35	7.71
20	5.94	17.86
40	5.35	15.89
80	4.02	20.13

[1] Each value represents a mean of 6 observations.

Statistical analyses similar to those applied to the data on heart weights were made on the data concerning liver weights. These analyses indicated that the livers of the pigs assigned the 10 ppm level of iron were significantly ($P \leq 0.05$) smaller per unit of body weight than those from pigs fed the higher levels of iron (table 2). The biological significance of this result is not readily apparent.

The estimate of 60 ppm of Fe obtained in this investigation is lower than that reported by Ullrey et al. ('60), who estimated the requirement as 125 ppm. The discrepancy between these estimates apparently is not directly correlated with rate of gain since the pigs receiving the adequate level of iron in this study gained about 0.34 kg per day as compared with 0.22 kg per day reported by Ullrey et al. ('60). The fact that the iron was added in both investigations on the basis of parts per million of the dry matter intake rather than milligrams of iron per day also diminishes the probability that rate of gain was a significant factor. Since ferrous iron sulfate was used in both studies, source of iron can be ruled out. Presumably, then, the differences in the requirement estimates are associated with the difference in dietary regimen, namely, the iron in the milk diet used in this study perhaps was more available than iron in the synthetic diet used by Ullrey et al. ('60). The reason for a possi-

ble difference in availability is not readily apparent. In view of the interfering effect of manganese on iron utilization by the baby pig reported by Matrone et al. ('59), knowledge of the actual manganese content of the synthetic milk diet might be informative.

It may be of interest to note that Mitchell ('47) working with the rat, which has a fast growth rate similar to that of the baby pig, obtained an estimate of 60 ppm of iron for the requirement of the rat.

Iron utilization

The iron utilized was considered to be that fraction of food iron appearing in hemoglobin synthesized during the experimental period. The validity of the basic assumptions inherent in the following calculations of the estimates of iron utilization has been discussed by Hahn et al. ('43), Drabkin ('51) and Matrone et al. ('57).

Specifically, the formula used to calculate the estimates of iron utilization is as follows:

$$\text{Fe utilization (U)} = \frac{3.34\left[\frac{TC_0}{C_x} - (BV_0 \times Hb_0)\right]}{\text{mg dietary Fe}}$$

where

3.34 = mg Fe/gm Hb
BV_0 = initial blood volume which was estimated to be 6% of initial body weight of a pig
Hb_0 = initial Hb value expressed as a fraction
C_0 = counts Fe^{59}/gm Hb on initial day
C_x = counts Fe^{59}/gm Hb on xth day
$TC_0 = (BV_0)\ (Hb_0)\ (C_0)$ or total Fe^{59} counts in Hb on initial day

C_0 was estimated by extrapolation of the straight line portion of the plot, log of counts/gm Hb versus log of weight × Hb. A typical plot is shown in figure 3. The plot is characterized by an initial curvature which presumably represents the phase of Fe^{59} incorporation into heme and a straight line portion which was assumed to be a direct function of dilution by unlabeled, newly formed hemoglobin. The intercept of the straight line extrapolation with the Y axis, determined by the method of least squares, was used as the initial specific activity of hemoglobin free of incorporation time (C_o). Thereafter, estimates of the total hemoglobin of a pig could be made each time a hemoglobin determination and radioactive count were taken. With these latter values the specific activity of the hemoglobin on day x was obtained (C_x). The total initial counts in hemoglobin (TC_o) were then divided by C_x to obtain the total hemoglobin in a pig on day x of the experiment.

Estimates of iron utilization obtained in this manner from experiments 1 and 2 are shown in table 3. It is apparent from the values in the table that depletion of the iron stores of the pig prior to initiation of the iron treatments did not enhance iron utilization since the average utilization values obtained in experiment 2, where no depletion was practiced, are higher ($P \leq 0.05$) than those obtained in experiment 1. This observation is similar to the results observed with calves (Matrone et al., '57).

Analysis of variance of these data indicated that the iron utilization values for the 40 and 80 ppm levels of iron were significantly lower ($P \leq 0.05$) than those for the 10 and 20 ppm levels. The utilization

TABLE 3

Estimates of utilization of dietary iron for hemoglobin synthesis by piglets

Experiment		Ppm of iron in diet[1]			
		10	20	40	80
		%	%	%	%
1		88[2]	39	36	23
		41	38	18	20
		29	—	—	19
	Mean	52.7	38.5	27.0	20.7
2		70	64	33	56
		48	55	39	32
		—	—	30	26
	Mean	59.0	59.5	34.0	38.0

[1] Moisture-free basis.
[2] Each value represents estimate from one pig.

values obtained for the 40 and 80 ppm levels within experiments, however, were not significantly different from each other. It would appear from the results with pigs not depleted that a rough approximation of the utilization of dietary iron for hemoglobin formation by the baby pig fed an adequate but minimal level of iron is about 30%.

Since it was possible to make estimates of iron utilization every 5 days of the experimental period, these values were analyzed for rate of change in percentage of utilization with time. An analysis of variance of the regression coefficients obtained for the lines fitted to the data by the method of least squares did not indicate significant differences among dietary levels of iron or between experiments. Although the pigs fed the higher levels of iron exhibited a small positive increase in iron utilization with time, decisive evidence was lacking that a signficant change in utilization occurred.

The specific activities expressed as counts per milligram of iron of the various excised tissues are shown in table 4. An examination of the data reveals two significant relationships: one concerning the effect of dietary iron level on specific activity, and the other concerning the relative specific activity of the tissues versus hemoglobin.

The tissues, as well as the hemoglobin obtained from the pigs fed the lowest level of iron, showed the highest specific activity. This result probably is a consequence of the smaller pool of total body iron in the pigs fed the lowest level of iron. However, if the dilution effect were the only factor operating, one would expect an inverse relationship between the specific activity and dietary levels of iron of the tissues. Although in fact this relationship holds for hemoglobin and bone marrow, it does not hold for the other tissues, since after the first decrease in specific activity, going from the 10 ppm level of iron to the 20 ppm, no further significant decrease is discernible. It is also interesting to note that the decrease in specific activity from the lowest to the second level of iron is greater in the tissues than in the hemoglobin. These data indicate that at the critical end of iron deficiency relatively more of the dietary iron is shunted to the tissues. This suggests that there is a minimum iron need required by the tissues which takes precedence over iron required for hemoglobin. Presumably this tissue iron might be that iron required for the iron enzymes and coenzymes concerned with the basic function of cell respiration.

The similarity of the specific activity of the tissues from pigs fed the 20, 40 and 80 ppm levels, however, suggests that once the basic needs for iron of the tissues are met the bulk of the absorbed iron goes directly to the hemoglobin pool with very little exchange with the tissue iron pool. Another interesting facet of these observations is that diverse tissues such as spleen,

TABLE 4

Average specific activity of various tissues

Dietary level of Fe	Fe^{59} counts/mg of iron					
	Liver	Spleen	Kidney	Muscle	Bone marrow	Hemoglobin
ppm						
			Experiment 1			
10 (3)[1]	5,185	4,798	6,741	2,922	15,781	27,138
20 (3)	1,038	1,336	1,826	963	1,710	12,241
40 (3)	684	1,299	1,531	1,275	1,080	7,834
80 (3)	533	1,032	787	991	685	5,912
			Experiment 2			
10 (3)	6,799	6,410	4,778	4,360	—[2]	15,389
20 (3)	654	7,019	1,749	2,750	1,618	7,479
40 (3)	1,537	1,926	1,649	1,646	930	4,896
80 (2)[2]	976	957	1,000	2,158	495	1,910

[1] Figures in parentheses indicate number of observations.

[2] Missing observations due to accidental breakage of sample tubes.

liver, kidney and muscle, in general, have specific activities of the same order of magnitude, suggesting that they belong to a common iron pool.

SUMMARY

Hemoglobin and weight-gain data from three experiments indicate that the minimum iron requirement of baby pigs, fed fortified cows' milk, up to 60 days of age was approximately 60 ppm of the dry matter intake. Estimates of the synthesized hemoglobin, made from these data and radioactivity data, indicate that utilization of the iron at the minimum requirement level was approximately 30%. Making baby pigs anemic (about 6 gm Hb/100 ml blood) by depleting them of iron before initiating iron feeding did not increase iron utilization. Specific activty data of blood, liver, spleen, kidney and bone marrow suggested that at the critical end of iron deficiency, tissue iron needs took precedence over hemoglobin iron need. Once this minimum tissue need was met, however, absorbed iron appeared to be incorporated directly into hemoglobin without equilibrating with tissue iron.

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A New Analogue of Vitamin B_{12} More Efficient for Growth Response of Rats

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We have recently isolated two analogues of vitamin B_{12} that are biosynthesized by a strain of *Nocardia rugosa* (Di Marco et al., '56), in the medium of which the 2,4,5-triamino-toluenetrihydrochloride is introduced as precursor. It has been shown (Di Marco, '59) that both of these analogues contain 5(6)amino-6(5)methylbenzaminazole, probably differing in position at point 5 or 6, respectively, of the amino group. Both are electropositive in 0.5 N acetic acid solution; but they are distinguishable by their different electrophoretic behavior.

Of the two analogues, that which is characterized by a slower mobility toward the cathode has been studied and in the following discussion we refer to this compound as "analogue." Like vitamin B_{12}, this substance promotes growth of microorganisms (Di Marco, '59). We report here studies of its growth-promoting effect in rats.

EXPERIMENTAL

Male albino rats of the Wistar strain, born to mothers maintained with the control diet, were used. The rats weighed from 60 to 70 gm and were fed a vitamin B_{12}-deficient ration (Wagle et al., '58). The analogue and vitamin B_{12} were administered in the diet to several groups of animals for 70 days at increasing concentrations of 25, 50, 100 and 200 μg per kg of diet. The control group received the unsupplemented diet. Food and water were supplied ad libitum and daily food intake was measured during the entire period.

The percentage composition of the diet follows: full-fat soya flour, 72; lactose, 22; salts,[1] 3; choline chloride, 0.5; DL-methionine, 0.1; and vitamins.[2]

Microscopic studies were carried out; sections of organs were fixed in formalin and slides prepared and stained with appropriate methods.

Where sufficient data were available, statistical analyses were made, using the usual procedures for the mean, standard error of the mean, Fisher "t" and the variance analysis for an assay at parallel lines and at 6 points (Burn et al., '50).

Vitamin content in serum and tissues was assayed microbiologically, according to the method of the U.S.P. ('55).

RESULTS

Body weights of the test animals were recorded at 20, 40 and 70 days, and the average of the initial and successive weights of all groups is shown in table 1. Both the analogue and vitamin B_{12} were observed to promote weight gain greater than that obtained in the control rats, and the differences have high statistical significance ($t > 2.75$ for $P = 0.01$) at 70 days, for levels higher than 50 μg per kg of diet.

The analogue, however, proved more efficient than vitamin B_{12} and the differences are statistically significant; in fact by the variance analysis we can deduce that the relative potency between the two substances, determined at the higher levels, is equal to 4.43 with fiducial limits of 8.64 and 2.27 for $P = 0.05$.

In table 2 are presented the fundamental elements of analysis and the variance ratio (F ratio). The F ratio, as expected, is highly significant for preparations, regression and doses, but not for parallelism, curvature and difference of curvatures. This means that a true difference exists be-

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[1] See Wagle et al., ('58).

[2] See footnote 1.

TABLE 1

Growth of rats fed vitamin B_{12}-deficient diet supplemented with analogue or vitamin B_{12}

Treatment	No. animals	Average weights (days)				Statistic "t"[1]
		0	20	40	70	
μg/kg diet		*gm*	*gm*	*gm*	*gm*	
None	49	70	92	111	127±4[2]	
Analogue-supplemented						
25	10	69	95	126	157±9	2.94
50	18	58	96	140	181±6	6.27
100	13	62	114	156	195±8	7.82
200	9	70	118	174	201±10	7.04
Vitamin B_{12}-supplemented						
25	4	69	83	118	147±6	1.54
50	8	66	91	132	157±8	3.20
100	7	68	98	132	161±3	3.61
200	8	70	108	152	184±4	6.51

[1] Fisher "t" to controls ("t" > 2.75 significant for P=0.01).
[2] Standard error of the mean.

TABLE 2

Analysis of variance of data in table 1 for the doses of 50, 100, and 200 μg of analogue and vitamin B_{12} (7 rats per treatment)

Adjustment for mean 1,377,772				
Nature of variation	d.f.	Sum of squares	Mean square	F ratio
Preparations	1	6168.595	6168.595	24.295[1]
Regression	1	3543.750	3543.750	13.957[1]
Parallelism	1	141.750	141.750	0.558
Curvature	1	3.440	3.440	0.013
Difference of curvatures	1	293.440	293.440	1.155
Between doses	5	10151	2030.2	7.92[1]
Error	36	9142	253.9	
Total	41	19293		

[1] Statistically significant for P=< 0.01.

tween the two substances and between doses, that there is a regression between doses and effects and that the two regression lines are parallel and straight.

The diet consumption was considered as average daily intake per 100 gm of body weight and as total intake per gram of final body weight gain. Both substances increased, in an almost equal degree, the daily intake (about 10%), and improved the coefficient of food utilization (about 25%) in respect to the control rats.

To supplement growth data, hematological measurements, weight and histological examinations of some organs and vitamin concentrations of some tissues were recorded also.

Both the analogue and vitamin B_{12} produced only a small increase in hemocytometric values above the control, which was not significantly abnormal. Both substances induced equally an increase (about 70 to 100%) of the relative weight of thymus, ventral prostate, seminal vesicles and levator ani muscle, and a decrease (about 20 to 50%) of thyroid, liver, kidneys, adrenals, spleen and testes and probably of the hypophysis.

The histological examination revealed some pathological changes in control rats, which consisted essentially of inflammatory processes in the form of small abscessed foci in the liver, lymphatic processes in the mesenterium, calcareous-like concretions, strongly stained with hematoxylin, formed in the lumen of the tubules in the juxtamedullary area (65%

Fig. 1 Sagittal section of the proximal end of a tibia in deficient rat. Reduced thickness of growth cartilage at the epiphyseal plate, scarce metaphysial trabecular pattern, decrease in thickness of the shaft. Hematoxylin and eosin stain. × 50.

of the cases), and hypoplasia of the testes, anterior prostate and seminal vesicles.

In addition, a marked hypotrophy and immaturity of the bone, due to a strong inhibition of the osteopoietic and remodeling processes, was present and concurrently a depression of chondrogenesis leading to the formation of a bone (tibia) with a thin diaphysis, scarce metaphysial trabecular structure and a strong reduction of epiphyseal cartilage (figs. 1, 2, 3).

Both analogue and vitamin B_{12} prevented, to an almost equal degree, the alterations of the sexual organs and bone tissue, and inflammatory lesions, but kidney lesions were not improved. Other organs examined (hypophysis, thyroid, parathyroid, adrenals, spleen and pancreas) were not affected.

The vitamin content of serum and tissues of treated rats (table 3), assayed microbiologically on some of the animals at the end of the experiment, was generally greater than that in control rats and proportional to the doses, except in the liver; equal doses of analogue and vitamin B_{12} produced about the same levels, with the exception that the serum concentration of the analogue was almost half that of vitamin B_{12}.

DISCUSSION

The new analogue of vitamin B_{12}, administered *per os* in the diet to deficient rats, has produced growth results greater than those of vitamin B_{12} itself. This greater activity is not due to an increase of food intake; the difference in food consumption between the two treatments was, in fact, negligible. Both substances similarly improved the coefficient of food utilization.

To obtain a severe vitamin B_{12} deficiency, we used a soybean meal-base diet that produces this effect without the use of drugs as, for example, thyroid hormone or sulfonamides (Wagle et al., '58); this ra-

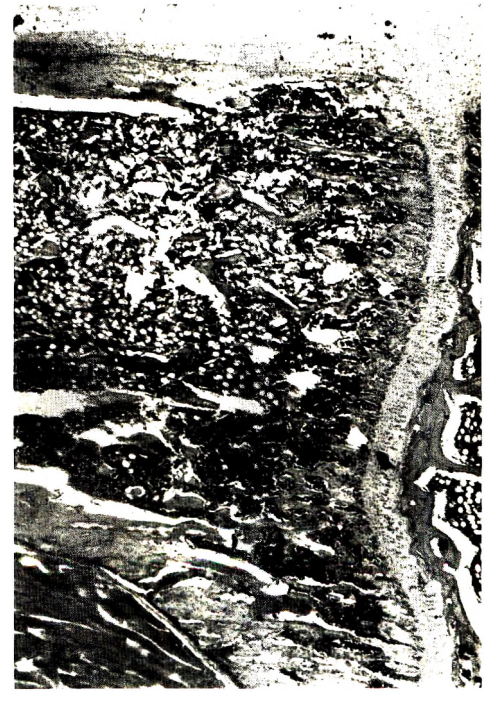

Fig. 2 Sagittal section of the proximal end of a tibia in deficient rat supplemented with analogue (50 μg/kg diet). Evident metaphysial trabecular pattern. Hematoxylin and eosin stain. × 50.

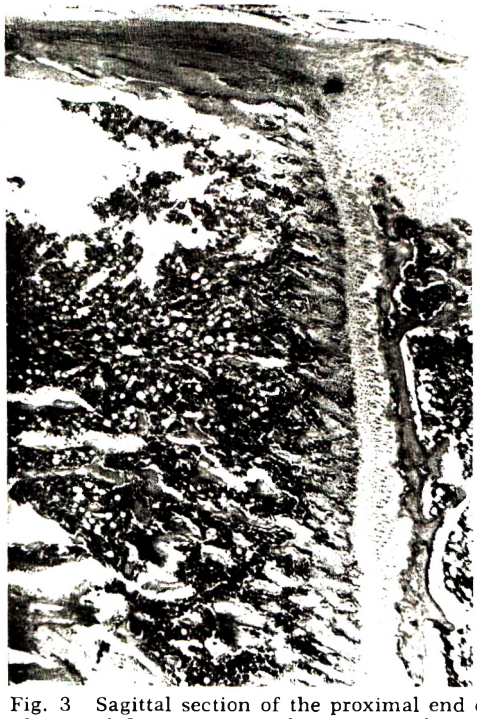

Fig. 3 Sagittal section of the proximal end of a tibia in deficient rat supplemented with vitamin B_{12} (50 μg/kg diet). Evident metaphysial trabecular pattern. Hematoxylin and eosin stain. × 50.

tion, however, induced severe histological alterations and it was not determined whether they were due only to vitamin B_{12} deficiency or to eventual and more complex factors, as a toxic action of soya flour (Borchers, '58), an altered balance of proteins, lipids and carbohydrates (Peterson and Register, '58)[3] or an alteration of water and Na, K, Ca and Mg ion metabolism due to an excess of lactose in the diet (Heggeness, '59). Both the analogue and vitamin B_{12}, in addition to their effect upon growth, prevented organ changes.

The tissue and serum concentrations of the vitamins obtained from rats treated by both substances were more elevated than those of control animals and demonstrate that both are well absorbed and distributed.

If we compare the concentrations obtained from 50 µg of analogue with those obtained from 200 µg of vitamin B_{12}, that is, the doses of both substances that induce about the same growth increase, we can observe that the analogue concentrations in serum, kidneys, spleen and thymus are about one-third those obtained from rats treated with vitamin B_{12}, but these values are almost equal in the liver.

From all these data we can conclude that the analogue is quantitatively more active than vitamin B_{12} in promoting growth, but it is not possible to deduce through which mechanism of action this effect is realized.

SUMMARY

The growth action of a new analogue of vitamin B_{12} isolated in our laboratory has been studied in rats fed a vitamin B_{12}-deficient soybean-base diet.

The analogue promoted much more efficient body weight gain in rats than vitamin B_{12}.

Diet intake, hematological picture and weight, histological aspects and vitamin content of some organs have also been considered: both substances modified behavior to an almost similar mode and degree.

It was not at once possible to deduce which mechanism of action makes the analogue more active than vitamin B_{12}.

[3] Hartman, A. M., and L. P. Dryden 1959 Effect of high levels of protein and of individual amino acids in vitamin B_{12} deficiency. Federation Proc., *18:* 529 (abstract).

TABLE 3

Serum and wet tissue concentrations of the analogue or vitamin B_{12} ($\mu g\ 10^{-3}/ml$ or gm) of treated rats

Treatment		No. of animals	Serum	Liver	Kidneys	Spleen	Thymus
µg/kg diet							
None		8	0.327	47.7	352	31.24	14
Analogue	50	3	0.640	99	594	51.00	4.1
Analogue	100	10	0.809	95.8	812	82.78	12.7
Vitamin B_{12}	100	3	1.765	105	700	78.00	14
Vitamin B_{12}	200	5	1.792	70.8	1400	196.6	74.8

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Proceedings of the Twenty-Fourth Annual Meeting of the American Institute of Nutrition

CONRAD HILTON HOTEL, CHICAGO, ILLINOIS
APRIL 11–15, 1960

COUNCIL MEETINGS

The American Institute of Nutrition Council met on Saturday evening, April 9, and on Sunday morning and evening, April 10. Formal actions of the Council were reported at the Institute business meetings and are included in the following minutes.

SCIENTIFIC SESSIONS

A full 5 days of scientific sessions on nutrition and related sciences were held as a part of the annual meeting of the Federation of American Societies for Experimental Biology. A total of 148 abstracts was submitted to the Institute for inclusion on the scientific program. Of these, 18 were transferred to the programs of other societies and 17 to intersociety sessions. With the 16 abstracts received by transfer from other societies, a nutrition program of 13 half-day sessions was arranged (and published in the March issue of *Federation Proceedings*, part 2). In addition, 4 half-day intersociety sessions on atherosclerosis were arranged under AIN sponsorship. Of special interest were 5 half-day symposia covering various nutritionally related subjects with invited speakers. All sessions were well attended with the symposia attracting up to 500 persons. All abstracts were published in the March 1960 issue of *Federation Proceedings*. Several of the symposia papers will be published in future issues of *Federation Proceedings*.

BUSINESS MEETINGS

Two business meetings were held. The one on April 12 was attended by about 200 members, and that on April 14 by about 140. The president, Dr. D. W. Woolley, presided at both meetings, held at 11 A.M. The following actions were taken:

I. Minutes of 1959

The minutes of the 1959 meeting, as published in The Journal of Nutrition, *69:* 95, September, 1959, were approved.

II. Election

The Secretary transmitted the sealed ballots to the Teller's Committee, Dr. M. K. Horwitt, chairman, and Dr. W. W. Cravens. At the second business meeting the Committee reported the election results from 326 ballots received, as follows:

Effective July 1, 1960:
President: Floyd S. Daft
President-Elect: Paul György
Secretary (three-year term): Arnold E. Schaefer
Councilor (three-year term): Ruben W. Engel
Effective May 1, 1960:
Editorial Board, JOURNAL OF NUTRITION (4-year term):
Gerald F. Combs
Ruth M. Leverton
George V. Mann
Max O. Schultze

The names of individuals with the 10 highest number of votes for suggested members of the Nominating Committee were submitted to the President. (See complete list of officers and committees at the end of these proceedings.)

III. Constitutional Amendments

By over two-thirds of all votes cast, the following amendments to the Constitution and By-Laws were adopted by mail vote (please refer to the September issue of *Federation Proceedings*, *18:* 794, 1959, for the former wording):

A. Article VII, as changed in three separate votes (by votes of 242 for, to 25 against, 276 for, to 5 against, and 260 for, to 19 against, respectively) now reads:

Article VII. Publications

Section 1. The American Institute of Nutrition designates *The Journal of Nutrition* as an official organ of publication and may designate other publications as official organs upon a two-thirds majority of ballots cast by the membership by a mail vote or at any annual meeting.

Section 2. In accordance with the expressed wish of The Wistar Institute of Anatomy and Biology, owner and publisher of *The Journal of Nutrition,* the American Institute of Nutrition shall nominate members of the Editorial Board for its official organ. A. The editorial management of *The Journal of Nutrition* shall be vested in an Editorial Board consisting of an Editor and 16 board members. B. The Editor shall be chosen by the Editorial Board to serve a term of 5 years beginning July 1 of the year in which he is chosen, and shall be eligible for re-election. The Editor shall have the power to appoint an assistant and such an appointee shall be called Associate Editor. C. Four members of the Institute shall be nominated by the Nominating Committee for membership on the Editorial Board each year to serve a term of 4 years and taking office May 1 of the year in which they are elected. D. Retiring members of the Editorial Board shall not be eligible for renomination until one year after their retirement.

B. By a vote of 276 for, to 7 against, Article IX has been changed to read as follows:

Article IX. Changes in Constitution and By-Laws

Section 1. Proposed changes in the Constitution and By-Laws must be sent in writing to the Secretary at least three months before the date of the meeting at which they are to be considered, and must be signed by at least three members. After Council approval of a proposed change the Secretary shall send a printed copy to each member with the ballot at least two weeks before the next meeting and shall notify all members of the voting procedure to be followed. By direction of the Council, members may vote by mail or by vote at an annual meeting.

Section 2. Whether voting is by mail or at the annual meeting, two-thirds of the votes cast shall insure passage.

(NOTE: The Constitution and By-Laws of the Institute are printed each year in the September issue of *Federation Proceedings.*)

IV. Membership Status

The secretary reported that as of April 1, 1960, there were 568 members in the Institute, 517 active, and 51 retired members. This is a net increase of 65 members since last year. There was one resignation during the year (R. E. Johnson, effective February 1, 1960).

Members present at the business meeting stood for a moment of silence in memory and in recognition of the following 10 members of the Institute who had passed away since April 1, 1959:

T. G. H. Drake, October 28, 1959
*Walter H. Eddy, September 28, 1959
Cyrus E. French, January 3, 1960
*James L. Gamble, May, 1959
Ernest Geiger, June 3, 1959
Arthur Knudson, September 25, 1959
*John R. Murlin, March 17, 1960
Harry Spector, September 13, 1959
Tom D. Spies, February 28, 1960
*Russell M. Wilder, December 16, 1959

* Charter members of the Institute.

Appropriate resolutions which had been received for deceased members were read and approved. Copies are on file in the secretary's office. The resolution read in honor of Dr. John R. Murlin, past president, treasurer, and secretary of the Institute, past editor of the Journal, and the person most responsible for the founding of the American Institute of Nutrition, is given below:

RESOLVED, That the American Institute of Nutrition, assembled at Chicago, Illinois, in its Annual Meeting, April 14, 1960, place in its minutes for permanent record this statement of deep regret and sorrow at the passing of one of its most distinguished members, John R. Murlin, and further

RESOLVED, That high tribute be paid to Dr. Murlin for his outstanding scientific accomplishments in nutrition science, particularly for his work in the biological evaluation of proteins, and for his pioneer studies in the field of diabetes. Dr. Murlin was the first editor of *The Journal of Nutrition* from 1928 to 1939, was the first secretary of the founding group in 1928, was secretary-treasurer in 1933 and served two terms as president, in 1934 and 1935. He was held in great esteem and respect by all of his associates in this Society and elsewhere.

V. New Members

A. *Active members.* The Council received 188 nominations for active membership, of which 179 were unanimously approved by members at the business meeting. Of these, 174 have accepted membership in the Institute, as follows:

NEW MEMBERS—1960*

William E. Abbott	Maclean J. Babcock
Anthony A. Albanese	Bobby D. Barnett
Thomas H. Allen	George P. Barron
Carl Alper	Charles H. Barrows, Jr.
Stanley R. Ames	Margaret W. Bates
Clarence B. Ammerman	Virginia A. Beal
Donald L. Anderson	William B. Bean
Richmond K. Anderson	George H. Beaton
Lewis R. Arrington	Moises Behar
Daniel L. Azarnoff	John M. Bell

Duane A. Benton
Ernest Beutler
Verle R. Bohman
Geoffrey H. Bourne
J. Edgar Braham
John R. Brobeck
Harry P. Broquist
John H. Browe
William W. Burr, Jr.
Lida M. Burrill

Jeptha E. Campbell, Jr.
William B. Castle
Charles C. Clayton
George E. Combs
John G. Coniglio
Jerome W. Conn
Frank C. Consolazio
William E. Cornatzer
David B. Coursin
Sylvia Cover
Leo V. Crowley

Richard Dam
William H. Daughaday
Philip H. Derse
Paul A. di Sant'Agnese
Mei Yu Dju
Vincent P. Dole
Clifford W. Duncan

Cecile H. Edwards
Lawrence B. Embry

William W. Faloon
Louis C. Fillios
Clement A. Finch
Samuel J. Fomon
Allan L. Forbes
Jeffrey H. Fryer

George J. Gabuzda
Seetha N. Ganapathy
Stanley M. Garn
Louise F. Gray
Morton I. Grossman
Miguel A. Guzman

William H. Hale
Samuel Halevy
David B. Hand
Roger G. Hansen
Robert H. Harms
Harold E. Harrison
W. Stanley Hartroft
James F. Hentges, Jr.
Victor Herbert
Robert W. Hillman
Jules Hirsch
Robert E. Hodges
Laurence M. Hursh
Dorris Hutchison

G. Watson James, III
Jozef Janicki
Henry D. Janowitz
Harold Jeghers
Karl R. Johansson
Ralph M. Johnson
Joseph A. Johnston

Robert M. Kark
Robert Kaye
Ancel Keys
Gerald Klatskin
Roman Kulwich

M. Eugene Lahey
M. Daniel Lane
Calvin A. Lang
Frederick W. Lengemann
Amos E. Light
Robert F. Light
Charles U. Lowe
Henry W. Loy
A. Leonard Luhby
Channing H. Lushbough

Ole J. Malm
Gilbert J. Mannering
Ernest G. McDaniel
George A. McLaren
H. C. Meng
Henry Menge
Susan B. Merrow
Venkata C. Metta
Elwyn R. Miller
R. K. Mishra
Peter R. Moore
Erwin H. Mosbach
Dorothy S. Moschette
John F. Mueller
M. Lois Murphy

Edward C. Naber
Sidney S. Negus
Talmadge S. Nelson
Chester A. Newhall

Kunio Okuda
Lacy R. Overby

Ernest M. Parrott
Paul L. Pavcek
Herbert T. Peeler
Esther F. Phipard
Donald E. Pickering
Irving C. Plough
Hans Popper
James M. Price

Robert G. Ravdin
Merrill S. Read
Clyde R. Richards
David Rittenberg
William D. Robinson
Oswald A. Roels
Lorene L. Rogers

Harish C. Saxena
S. Stephen Schiaffino
Robert F. Schilling
David Seligson
Raymond F. Sewell
Frederick E. Shideman
William Shive
Arthur J. Siedler
Milton Silverman
Robert J. Sirny
Gladys A. Sperling
Jeremiah Stamler
Marian E. Swendseid

Edward J. Thacker
Rollin H. Thayer
Ethel M. Thompson
Samuel W. Thompson
Paul A. Thornton
Allen D. Tillman
David A. Turner

Duane E. Ullrey
Jerome A. Uram

Bert L. Vallee
Jan van Eys
Mitchell G. Vavich
Richard W. Vilter
Willard J. Visek

Max Wachstein
Bernice K. Watt
Johnnie H. Watts
Leslie T. Webster, Jr.
G. Donald Whedon
Priscilla M. Wheeler
Julius White
Frank Whiting
Jesse N. Williams, Jr.
Robert H. Williams
James R. Wilson
Maxwell M. Wintrobe

Richard S. Yamamoto

* For institutional affiliations and addresses of new members see the September issue of *Federation Proceedings*.

B. *Honorary members.* As a result of the change in by-laws adopted last year, the following 10 distinguished nutritionists, not members of the Institute, were nominated by the Council for honorary membership. All were approved by unanimous vote by the members and each person has since accepted. This is the first group so selected by the Institute.

HONORARY MEMBERS

W. R. Aykroyd, England
Frank B. Berry, United States
Frank G. Boudreau, United States
Robert C. Burgess, Switzerland
F. W. A. Clements, Australia
David P. Cuthbertson, Scotland
Lord John Boyd Orr, Scotland
V. N. Patwardhan, India
Emile F. Terroine, France
Artturi I. Virtanen, Finland

Thus, as of April 15, 1960, total membership of AIN stood at 751 members, including the 10 honorary members.

VI. *Treasurer's Report*

The report of the treasurer, J. B. Allison, from April 9, 1959 to April 8, 1960, as summarized on page 476 was read and approved.

The auditing committee, M. Wight Taylor and Walther H. Ott, submitted a report that the treasurer's accounts were correct and complete. Their report was approved.

Dues of $2.00 for the coming year were approved (no change from preceding year). The last installment of the special assessment of $2.00 per year for the International Congress on Nutrition, as approved in 1958, will be due this year.

Balance brought forward		$ 1,902.25
Receipts		
Institute dues from 520 members at $2.00	$1,040.00	
Federation assessments, 522 at $4.00	2,088.00	
Subscriptions to *Journal of Nutrition*, 505	4,290.50	
Assessments for Fifth International Congress on Nutrition, 523 at $2.00	1,046.00	
Grants and contributions to Fifth International Congress on Nutrition	1,019.50	
Interest on bond	13.80	
Nutrition dinner and smoker, 1959	162.69	
Wistar Institute, for editorial office	9,000.00	
AIN share of 1959 Federation registration	795.00	
Bank premiums on 3 Canadian checks	1.96	$19,457.45
Total received		$21,359.70
Expenditures		
Federation office, dues and expenses	$2,145.99	
Wistar Institute, subscriptions and printing	4,446.26	
Fifth International Congress on Nutrition	2,065.50	
Secretary's office	350.00	
Treasurer's office	100.00	
Editor's office, Cornell University	9,000.00	
Career leaflet	11.40	
President's office	13.14	
Bank charges	6.88	
Total expenditures		$18,139.17
Balance on hand, April 8, 1960		3,220.53
U. S. bond		500.00
TOTAL BALANCE		$ 3,720.53

VII. Editor's report—The Journal of Nutrition

The editor, Dr. Richard H. Barnes, submitted his report covering July 1, 1959 to March 15, 1960. It was approved and is summarized below:

Two meetings of the editorial board were held in 1959 in New York. Manuscripts received have increased in number by about 25 per cent. The rejection rate continues to be approximately 16 per cent. Thus, about a 25 per cent increase in the number of published papers can be expected in 1960. The elapsed time between receipt of a paper and its publication has been about 6 months, but the editors are striving for a 5 months' total publication time (which is expected to be reached this summer). The Wistar Press is currently operating on a three months' processing time. There is no backlog of papers. Volumes 67, 68 and 69 of the *Journal* were published in 1959. The change in format, the new cover design and smaller type, was made starting with volume 69 in September, 1959. The total cost of operation in the editor's office in the 8½-month period was $5,298.31.

VIII. Reports of Committees and Representatives

A. *Public Information Committee:* R. W. Engel, chairman.

Dr. Engel reported that approximately 3,000 copies of the leaflet, "Career Opportunities in Nutrition," were distributed during the year in response to individual requests. A revision of the leaflet will be made to include the addresses of related associations with additional career information.

The Federation has obtained the services of Dr. S. S. Negus to improve the public information services in connection with the Federation meeting. Dr. Engel stated that there was more effective news coverage of the meeting this year than of any previous meeting as the result of Dr. Negus's services.

The committee handled numerous requests during the year for information on specific aspects of nutrition. In many instances individual Institute members have

been called on to answer inquiries outside of the professional competence of committee members. In some instances the Food and Nutrition Board and the Food and Nutrition Council of AMA have been called on. The committee is appreciative of the assistance it has obtained.

The report was approved.

B. *Organizing Committee of the Fifth International Congress on Nutrition:* Dr. György gave a progress report on the plans for the Congress to be held in Washington, D. C., September 1–7, 1960. An enrollment of 2,000 persons or more is expected and good progress is being made with all aspects of the planning. The final meeting of the organizing committee was held on April 12. Final plans and details will be completed by the executive committee, the various committees and sub-committees, and by the secretariat, Dr. M. O. Lee.

C. *Representative to Division of Biology and Agriculture of the National Research Council:* N. R. Ellis.

Dr. Ellis's complete report is on file. He attended various meetings of the Food and Nutrition Board and the Agricultural Research Institute and handled a number of special requests for advice and information in this connection. He mentioned the availability of NAS-NRC publication, no. 711, "The Evaluation of Protein Nutrition" (prepared by the committee on amino acids); publication no. 654, entitled "Food Packaging Materials—Their Composition and Uses"; publication no. 646, "The Safety of Polyoxyethylene Stearate for Use in Foods"; publication no. 749, "Problems in the Evaluation of Carcinogenic Hazard from the Use of Food Additives"; and publication no. 750, "Principles and Procedures for Evaluating the Safety of Food Additives."

The Committee on Animal Nutrition has recently published a revision of publication no. 648, "Nutrient Requirements of Swine"; of publication no. 659, "Tables of Feed Composition"; and of publication no. 714, "Hormonal Relationships and Applications in the Production of Meats, Milk, and Eggs."

The office of the Handbook of Biological Data was transferred in September, 1959, to the Federation under the administration of Dr. M. O. Lee. Four volumes appeared in 1959 on circulation, insecticides, tranquilizers and fungicides.

The report was approved.

D. *Representative to AAAS Council, Section N-Medical:* Dr. Paul L. Day.

Dr. Day's report, which pointed out the association's plans for possible reorganization of the Council and committee structure, was approved.

E. *Ad Hoc Committee on Journal of Nutrition Management:* W. J. Darby, chairman:

Dr. Darby reported that in one more year the present contract with The Wistar Institute would be open for renegotiation, if necessary. In the next year the committee will re-evaluate the *Journal* situation, the contract with The Wistar Press, and possibly suggest any constitutional changes which may be necessary for improvement in our *Journal* situation.

F. *Representatives to Federation Board:* F. S. Daft, chairman:

Dr. Daft summarized the activities of the Federation Board and its advisory committee. Some of the problems which the board has been considering are a new building on the Federation grounds, public information activities, changes in *Federation Proceedings* publication policies, improved procedures in programming of the Federation meeting and meeting sites. Dr. Daft announced that the next meeting of the Federation would be in Atlantic City, April 10–15, 1961.

IX. Report of AIN Council actions—AIN divisions

The Council met on October 31, 1959, in Washington, D. C., and on April 9 and 10, 1960, in Chicago. Dr. Woolley reported that the primary concern of the Council at these two meetings was the possible formation of a division, within the framework of the Institute, devoted to clinical nutrition. Considerable thought was given to this proposal and the following proposed changes in the constitution and by-laws were formulated and unanimously approved by the Council.

ARTICLE IX *Divisions of the American Institute of Nutrition*

Section 1. With the approval of the Council there may be formed individual divisions within the framework of the American Institute of Nutrition. Members of each division shall be members of the Institute selected according to Article I of the Constitution. Within these limitations each division is empowered to determine its qualifications for membership and to select its own members.

Section 2. Such divisions will elect their officers within the framework of their own constitution. Each division shall have representation on the Nominating Committee of the American Institute of Nutrition. This Committee will take such account of the membership and structure of the Institute as to assure continuing representation of the division on the Council of the American Institute of Nutrition.

Section 3. Divisions of the Institute may hold separate meetings as they choose. They may also publish or sponsor the publication of a journal(s) as an official organ(s) of that division. The editorial board(s) of such publication(s) will be selected by the division according to its constitution. Contractual agreements concerning such publication(s) may be entered into by the individual divisions with the approval of the Council of the American Institute of Nutrition.

Section 4. Members of divisions of the American Institute of Nutrition will be assessed the basic annual assessment of the American Institute of Nutrition, the assessment of the Federation, divisional dues as determined by the individual divisions, and subscription to one official publication of the American Institute of Nutrition. In addition to the journal selected as obligatory, each member of a division may subscribe to any other official journal of the American Institute of Nutrition at the prevailing rate for members.

Section 5. The initial division to be organized will be a division of clinical nutrition.

After a complete explanation of the various reasons behind this proposal by Dr. Woolley and other members of the society, a test vote was taken by show of hands on the proposal as an expression of opinion. The test vote indicated general approval of the proposal (126 for, 14 against).

(The proposed amendment was voted on by mail ballot in July, 1960 and the proposal was carried by a vote of 460 for, to 21 against.)

X. Acknowledgments

The Secretary wishes to gratefully acknowledge the excellent secretarial assistance of Mrs. Nancy Hess, Mrs. Dorothy Clark and Miss Judi Tessitor and the staff of the Federation office for many hours of devoted help in connection with Institute activities.

ANNIVERSARY BANQUET AND PRESENTATION OF FELLOWS AND AWARDS

The annual banquet was held on April 13 at the Conrad Hilton Hotel with 330 members and guests in attendance. Dr. D. W. Woolley, as toastmaster, introduced the special guests and awardees.

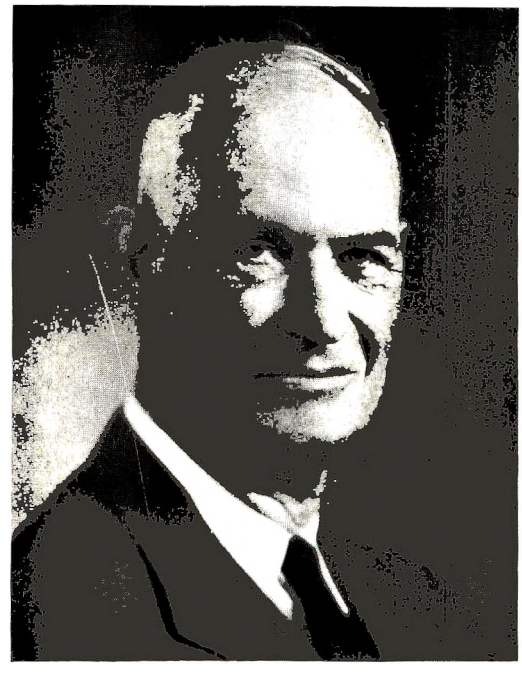
Dr. Cosmo Mackenzie, chairman, Committee on Fellows, presented Certificates of Fellow to the following persons selected in 1960 who have had distinguished careers in nutrition:

ICIE GERTRUDE MACY-HOOBLER

"For her classical studies on nutrition during pregnancy and lactation and in infancy and childhood which have contributed substantially to the betterment of mankind."

LEONARD AMBY MAYNARD

"For his investigations in the fields of animal and human nutrition, his studies on lipid metabolism, his leadership in the nutritional councils of the nation, and his guidance and stimulation of students at the undergraduate and graduate levels."



PAUL EDWARD HOWE

"For the development of methods for the analysis of plasma proteins, for his studies on nitrogen metabolism and the nutritional requirements of man and other animals, and for his contribution to the nutrition of the United States Army in World War II."

R. GAURTH HANSEN

NEVIN SCRIMSHAW

The 1960 *Borden Award in Nutrition* of $1,000 and a gold medal was presented to Dr. R. Gaurth Hansen of Michigan State University for his highly significant research contributions relating to the formation and utilization of galactose in animals and man and for other outstanding studies in intermediary metabolism and nutrition.

The 1960 *Osborne-Mendel Award* of $1,000 and a scroll was presented to Dr. Nevin Scrimshaw of INCAP, Guatemala, in recognition of "his numerous contributions to the understanding of protein and iodine deficiency in man."

After the banquet, the joint Biochemistry-Nutrition Smoker was held at the Conrad Hilton Hotel with approximately 800 present. The very efficient work of Dr. Bernard Schweigert in making the smoker a success is gratefully acknowledged.

OFFICERS and COMMITTEES—AMERICAN INSTITUTE OF NUTRITION

July 1, 1960 — June 30, 1961

Council

President: F. S. Daft, NIAMD, National Institutes of Health, Bethesda, Maryland
President-Elect: P. György, Philadelphia General Hospital, Philadelphia, Pennsylvania
Past-President: D. W. Woolley, Rockefeller Institute, New York City
Secretary: A. E. Schaefer, Building 16A, National Institutes of Health, Bethesda, Maryland (1963)
Treasurer: J. B. Allison, Rutgers, the State University, New Brunswick, New Jersey (1962)
Councilors: J. H. Roe (1961), W. H. Griffith (1962), R. W. Engel (1963)

Committees

Nominating Committee: J. M. Hundley, chairman, C. A. Baumann, Grace Goldsmith, C. G. King, L. D. Wright
Committee on Nomenclature (Joint with American Society of Biological Chemists): O. L. Kline, chairman (1961), P. L. White (1962)
Committee on Journal of Nutrition Management (ad hoc): W. J. Darby, chairman, P. György, O. L. Kline, H. A. Schneider
Nominating Committee for Borden Award: G. V. Mann, chairman (1961), E. E. Snell (1962), G. M. Briggs (1963)
Nominating Committee for Osborne-Mendel Award: J. S. Dinning, chairman (1961), Grace A. Goldsmith (1962), L. C. Norris (1963)
Fellows Committee: W. D. Salmon, chairman, (1961), Karl Folkers (1961), E. W. McHenry (1962), A. G. Hogan (1963), W. H. Sebrell, Jr. (1963)
Committee on Membership (ad hoc): P. György chairman, H. R. Bird, V. H. Cheldelin, R. E. Olson, E. N. Todhunter
Public Information Committee: P. L. White, chairman (1962), R. W. Engel (1961), S. S. Negus (1961), L. Voris (1962), A. E. Schaefer (*ex officio*) (1963)
Auditing Committee: W. H. Ott, chairman (1961), H. W. Titus (1961)
Tellers Committee: B. F. Chow, chairman (1961), D. V. Frost (1961)

U. S. National Committee—IUNS

Paul György, chairman (1961), W. H. Sebrell, Jr., vice-chairman (1961), R. W. Engel, secretary (1963), E. L. Severinghaus (1961), Gladys Emerson (1962), J. M. Hundley (1962), A. E. Schaefer (1962), G. M. Briggs (1963), L. J. Teply (1963)
Also, *ex officio* (voting) members are F. S. Daft (1961), Grace Goldsmith, C. G. King (1961); and *ex officio* (non-voting) members are H. B. Steinbach, R. K. Cannan, W. W. Atwood, Jr., and E. V. McCollum

Representatives to other organizations

Federation Board: D. W. Woolley (1961), F. S. Daft (1962) and Paul György (1963)
Federation Advisory Committee: F. S. Daft (1963)
National Research Council boards and divisions: N. R. Ellis (1963)
American Association for the Advancement of Science: E. L. Hove (Section C—Chemistry) (1962), P. L. Day (Section N—Medical) (1961)
Food and Agriculture Organization: L. J. Teply (1962)

Officers, American Society for Clinical Nutrition (a division of the American Institute of Nutrition): R. W. Vilter, president; R. E. Olson, president-elect; R. E. Hodges, University of Iowa School of Medicine, Iowa City, Iowa, secretary-treasurer; councilors—W. B. Bean, R. S. Goodhart, W. H. Krehl

Editorial Board, Journal of Nutrition: R. H. Barnes, editor (1964), R. W. Engel (1961), P. L. Harris (1961), H. A. Schneider (1961), O. L. Kline (1962), E. S. Nasset (1962), H. Pollack (1962), D. V. Frost (1963), A. E. Harper (1963), O. Mickelsen (1963), G. F. Combs (1964), R. M. Leverton (1964), G. V. Mann (1964), M. O. Schultze (1964)

Respectfully submitted,
GEORGE M. BRIGGS, *Secretary*
American Institute of Nutrition

ERRATUM

HALEY, E. E., AND J. P. LAMBOOY 1960 The biological activity of 6-chloro-7-methyl-9-(1′-D-ribityl)-isoalloxazine. J. Nutrition, *72:* 169.

(Ms. error) On page 170, the first paragraph under METHODS *should have read as follows:*

METHODS

Acid production by L. casei. The quantity of lactic acid produced by *L. casei* 7469[4] was measured by titration with 0.1 N sodium hydroxide. The observed values were plotted against the appropriate flavin concentrations. One set of duplicate tubes was prepared by the routine procedure (Association of Vitamin Chemists, '51) using graded increments of riboflavin (USP reference standard) from zero to 0.3 μg per tube. Another set of duplicate tubes was prepared to contain in all cases 0.3 μg of riboflavin per tube plus graded increments of 6 - chloro - 7 - methyl-flavin from zero to 100 μg per tube. All incubations were for 72 hours at 37°C. The inhibition index was determined from the ratio of the mixture of the two flavins which supported the formation of one-half the amount of lactic acid formed in the presence of the same amount of riboflavin alone.[5] This method for the determination of the inhibition index has been used by Woolley ('44) for the evaluation of the activity of a phenazine compound.

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SYMPOSIUM ON MAMMALIAN GENETICS AND REPRODUCTION

Sponsored by

THE BIOLOGY DIVISION

OAK RIDGE NATIONAL LABORATORY

The papers originating from this Symposium are made available to scientific circles for the first time as a Supplement issue to the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY. The publication is identified as *Supplement 1, volume 56, Journal of Cellular and Comparative Physiology*, and is available without added cost to subscribers for 1960 volumes 55–56 and if requested with new subscription orders for the 1961 volumes 57–58. Single copies of Supplement issues are not for sale but are available only to subscribers of the journal. *Information concerning reprints may be obtained by addressing The Biology Division, Oak Ridge National Laboratory.*

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