Metabolic Patterns in Preadolescent Children

V. INTAKE AND URINARY EXCRETION OF PANTOTHENIC ACID AND OF FOLIC ACID[1]

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Schmidt ('49–'51) has reported on the urinary excretion of pantothenic acid by children but there are no reports in the literature on folic acid excretion of children. Likewise no literature reference was found on amounts of each of these vitamins in mixed diets of ordinary foods commonly eaten by children in this country.

The pantothenic acid and folic acid content of controlled diets and the urinary excretion of these vitamins were determined for 35 healthy girls, 7 to 9 years of age, as one segment of a series of metabolic studies carried out with multinutrient analyses. A detailed description of the studies conducted in the three years is given in the Southern Cooperative Series Bulletin no. 64 ('59).

The 1954 study with 11 girls participating consisted of 16 4-day periods, the 1956 study with 12 girls of 14 4-day periods, and the 1958 study with 12 girls of 8 6-day periods. In the 1958 study, assays for pantothenic acid and folic acid were done on food and on urine composites for each period; whereas in the two earlier studies assays for these vitamins were carried out on composites for alternate periods.

The characteristics of the groups of girls and the diets for the three metabolic studies are shown in table 1. The mixed diets were planned to provide nutrients at levels recommended for 7- to 9-year-old children (National Research Council, '53) except for protein which was the variable. Energy and nitrogen determinations were made on each of the diets and these results have been reported (Moschette, '60; James, '60).

Because metabolism was studied at several levels of protein, it was possible to ascertain whether the excretions of these vitamins bear any relation to nitrogen retention or to other metabolic characteristics of 7- to 9-year-old girls. Pantothenic acid and folic acid intakes were not held constant but varied with kinds and amounts of foods included in the diets.

PANTOTHENIC ACID

Because pantothenic acid is widely distributed in foods there is little danger of a dietary deficiency of this vitamin alone. If a deficiency occurs in human beings, it would likely occur in combination with other vitamin B-complex deficiencies. On the basis of data reported in the literature, Ralli ('54) estimated that the approximate pantothenic acid intake in the United States is from 3 to 12 mg per day and that the daily requirement for adults is from 3 to 5 mg.

Results and discussion. In the studies reported here the amount of pantothenic acid in the diets and the amount excreted in the urine were determined by microbiological assay with *Lactobacillus plantarum* (Zook et al., '56). The food composites were incubated with pigeon liver and intestinal phosphatase for release of

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bound pantothenic acid. Urine was assayed without the enzyme treatment, preliminary investigation having indicated that this was not necessary.

The average daily pantothenic acid intake was 4.49 mg for the 11 girls studied in the fall of 1954, 5.00 mg for the 12 girls studied in the summer of 1956, and 2.79 mg for the 12 girls studied in the summer of 1958 (table 2). The average daily urinary pantothenic acid and the percentage of the intake excreted were 2.85 mg (63%), 1.71 mg (34%), and 1.31 mg (47%) for the three years, respectively.

The 1954 study was carried on in the fall while the girls were attending school; whereas the 1956 and 1958 studies were conducted in the summer and therefore permitted more time for activity and recreation. Subjects in the 1954 study had the highest urinary pantothenic acid, although their average intake was 10% less than in 1956. In general, with the higher

TABLE 1

Intakes of protein and energy, and ranges in initial body weights for 35 girls receiving controlled diets

Year and subject code no.[1]	Diet no.[1]	Protein intake[2]	Energy intake[3]	Initial body weight
		gm/day	*Cal./day*	*kg*
1954—11 girls				
1–11	1	65	1955	22.4–42.7
1956—12 girls				
12–14(S)[4]	2	48	1948	22.1–25.0
15–17(S)	3	73	1966	26.3–27.4
18–20(L)	4	59	2356	29.9–31.4
21–23(L)	5	88	2415	31.4–33.6
1958—12 girls				
24–35	8	22	2177	21.9–34.1
24–35	8′[5]	18	2240	23.0–34.1

[1] Subject and diet numbers correspond with those in Southern Cooperative Series Bulletin no. 64 ('59).

[2] Kjeldahl nitrogen × 6.25.

[3] Energy, determined in an Emerson bomb calorimeter, represents gross energy or heat of combustion due to the oxidation of the food to its ultimate oxidation products.

[4] Small girls (S); large girls (L).

[5] Diet 8′ was essentially the same as diet 8 except that milk was omitted to reduce the protein content.

TABLE 2

Average daily pantothenic acid intake and urinary excretion determined by microbiological assay with L. plantarum

Year and subject code no.[1]	Diet[1]	Intake		Urinary excretion		
		mg	*S.D.*[2]	*mg*	*S.D.*	*% intake*
1954—11 girls						
1–11	1	4.49	0.76	2.85	0.60	63
1956—12 girls						
12–14	2	4.17		1.42		34
15–17	3	5.41		1.99		37
18–20	4	4.80		1.58		33
21–23	5	5.64		1.88		33
12–23		5.00	0.82	1.71	0.57	34
1958—12 girls						
24–35 (30 days)	8	3.01		1.44		48
24–35 (15 days)	8′	2.42		1.09		45
24–35		2.79	0.33	1.31	0.28	47

[1] Subject and diet numbers correspond with those in Southern Cooperative Series Bulletin no. 64 ('59).

[2] Standard deviation of the observations.

intakes of pantothenic acid the urinary excretion tended to be higher.

No definite trends in urinary excretion of the vitamin were noted for either the 1954 or 1956 studies with girls receiving constant daily intakes of 4.49 and 5.00 mg, respectively. There was considerable variation in excretion among girls and among periods.

In figure 1 is shown the trend in the urinary pantothenic acid with two lower levels of intake, namely, 3.01 and 2.42 mg per day, for the 12 girls studied in 1958. The average amount excreted was highest for the two-day preperiod when the girls first received the controlled diet. There is a definite trend toward lower excretion levels in successive periods with a daily intake of 3.01 mg and an indication that equilibrium was reached at about 20 days. Upon further reduction in intake to 2.42 mg, the average excretion of the vitamin again decreased in successive periods.

The diets that supplied the most protein also supplied the most pantothenic acid. In the 1956 study the diets containing 73 and 88 gm of protein supplied approximately 20% more pantothenic acid than the diets containing 48 and 59 gm of protein. In the 1958 study when the protein intake was decreased from 22 gm to 18 gm (18%) in periods 6 to 8, the pantothenic acid intake was decreased 0.59 mg (20%) and the average of the pantothenic acid excretions was decreased 24%. This relationship of pantothenic acid intake, and thus pantothenic acid excretion, to protein intake is a result of the foods high in protein also being high in pantothenic acid content. The amount of pantothenic acid excreted in the urine was not associated with the amount of nitrogen retained.

Urinary excretion of pantothenic acid could not be related to body size. Small girls (22.1 to 27.4 kg) excreted an average of 1.42 mg of pantothenic acid and the large girls (29.9 to 33.6 kg) excreted an average of 1.58 mg per day when receiving the lower protein diets in 1956. With the higher protein diets the small girls excreted an average of 1.99 mg of pantothenic acid per day and the large girls excreted an average of 1.88 mg per day.

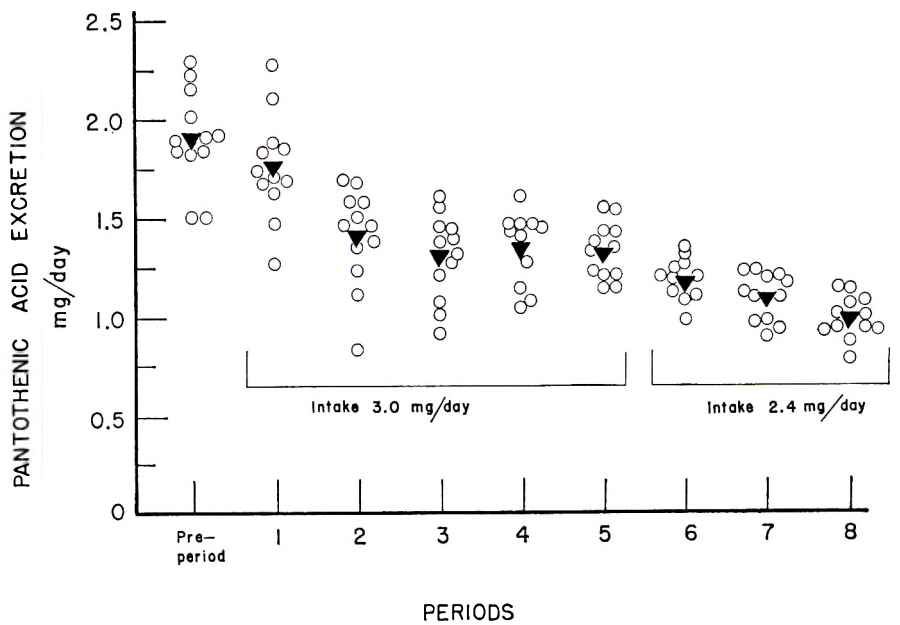

Fig. 1 Urinary pantothenic acid for 12 girls receiving controlled diets for a two-day preperiod and 8 consecutive 6-day periods. The symbol (○) refers to the average excretion for each girl and the symbol (▲) to group averages.

In table 3 values are given from reports in the literature on intake and urinary excretion of pantothenic acid for comparison with results obtained in these studies. Studies on urinary pantothenic acid excretions by adults gave averages of from 1.0 to 6.0 mg per day. In two studies Schmidt ('49–'51) found average urinary excretions of pantothenic acid of 2.05, 2.86, and 2.5 mg per day by children. In the study reported here the average value of 2.85 mg of pantothenic acid excreted by the girls in 1954 is comparable to the values obtained by Schmidt. Urinary excretions on the 1956 and 1958 studies of 1.71 and 1.31 mg per day are definitely lower, however, than any reported in the literature.

Gordon ('42), in studies with normal adults and adults with a general nutritional deficiency, found that an excretion of 1.0 mg per 24 hours appeared to be a rough dividing line between normal and deficient subjects, but that there was some overlapping of values.

Fox et al. ('59) in studying the metabolic response of young women observed that urinary pantothenic acid excretion responded to changes in the pantothenic acid content of the diet. Denko et al. ('46b) reported that urinary excretion of pantothenic acid dropped quickly with a restricted diet and that it was restored to levels of the control period by crystalline vitamin supplements.

FOLIC ACID

Harris and Thimann ('47) estimated from comparative data that the maintenance dose of folic acid for man is from 0.1 to 0.2 mg per day. Denko et al. ('46a) reported an intake of 0.043 to 0.086 mg with an average of 0.062 mg per day for adults consuming a weighed standardized diet of ordinary foods. Odland[2] reported an intake of 0.4 mg per day for young women consuming controlled diets.

[2] Odland, L. M., and H. T. Parsons 1950 Folic acid metabolism in normal human subjects. Federation Proc., 9: 367 (abstract).

TABLE 3

Values for pantothenic acid intake and urinary excretion reported in the literature and from these studies

Study	Subjects	Daily pantothenic acid	
		Intake	Urinary excretion
		mg	*mg*
Denko et al., '46a	7 young men	4.73[1]	3.04
Denko et al., '46b	5 young men	1.1[1]	1.0
Fox et al., '59	8 college women	2.8[2]	3.2
		7.8[2]	4.5
		12.8[2]	5.6
Gardner et al., '43	3 young women	6.9[1]	6.0
Gordon, '42	40 medical students or hospital staff		3.52
Oldham et al., '46	Young women	2.1–4.8[1]	1.6–4.0
Schmidt, '49	10 children, 1–10 years		2.05
	10 children, 7–14 years		2.86
Schmidt, '51	17, 2–14 years		2.5
	56, 16–45 years		2.7
	23, 51–82 years		2.3
These studies, '54	11 girls, 7–9 years	4.49[2]	2.85
'56	12 girls, 7–9 years	5.00[2]	1.71
'58	12 girls, 7–9 years	2.79[2]	1.31

[1] Food analyses were made without use of the double enzyme system for liberation of bound pantothenic acid; therefore, values probably do not represent total pantothenic acid intake.

[2] Double enzyme system used to determine total pantothenic acid.

Results and discussion. In the studies reported here the amount of folic acid furnished by the diets and the amount excreted in the urine were determined by microbiological assay as described by Toepfer et al. ('51). Food composites were incubated with chicken pancreas for release of bound folic acid. The folic acid content of urine was determined on composites without enzyme treatment. Two micro-organisms, *Lactobacillus casei* and *Streptococcus faecalis*, were used for determinations on the 1954 and 1956 samples. Only *S. faecalis* was used in the analysis of all of the samples in the 1958 study; however, some unusually low values for urine were checked with *L. casei*.

Assays for the content of folic acid in food composites gave values with *L. casei* which were from 29 to 42% lower on the average than those obtained with *S. faecalis*. Assays on urine in the 1954 study gave comparable results with the two organisms, whereas in the 1956 study, values on urine obtained with *L. casei* averaged from 25 to 40% lower than those obtained with *S. faecalis*. Values for food and urine in the 1958 study were determined with *S. faecalis* only.

The average daily folic acid intake in each of the three studies as determined with *S. faecalis* was 98, 80, and 52 μg, respectively, and correspondingly the average daily urinary excretion was 1.24, 1.13, and 1.41 μg (table 4). These values are comparable with those of 43 to 86 μg with constant weighed diets reported by Denko et al. ('46a) using *S. faecalis* as the test organism.

The amount of folic acid excreted in the urine was small as compared with the intake. The approximate mean percentages excreted by the girls were as follows: in 1954, 1%; in 1956, 1%; and in 1958, 3%. The amount of folic acid excreted did not show relationship to folic acid intake, pantothenic acid intake, protein intake, or body weight.

Odland ('50) reported a urinary excretion of less than 1% of the dietary intake of folic acid with intakes of 400 μg. Denko et al. ('46a, b) found that the amount of folic acid in the diet had little effect on the amount excreted in the urine. With an intake of 62 μg the excretion was from 3 to 5 μg per day, or approximately 6% of the intake; with a restricted diet containing 22 μg of folic acid, the excretions averaged 3 μg per day, or 14% of the intake.

Urinary excretions of folic acid for adults reported in the literature and for 7- to 9-year-old girls as found in the present studies are given in table 5. In literature reports, excretions for adults consuming normal diets averaged from 2.32 to 10.8 μg per day as assayed with *S. faecalis*.

TABLE 4

Average daily folic acid intake and urinary excretion as determined with two micro-organisms

Year and subject code no.[1]	Diet[1]	Intake				Urinary excretion			
		L. casei		*S. faecalis*		*L. casei*		*S. faecalis*	
		μg	S.D.[2]	μg	S.D.	μg	S.D.	μg	S.D.
1954—11 girls									
1–11	1	67	11	98	14	1.26	0.44	1.24	0.33
1956—12 girls									
12–14	2	46		79		0.85		1.07	
15–17	3	50		76		0.86		1.05	
18–20	4	46		80		0.74		1.07	
21–23	5	53		85		1.01		1.32	
12–23		49	6	80	10	0.86	0.50	1.13	0.30
1958—12 girls									
24–35 (30 days)	8	—		50		—		1.41	
24–35 (15 days)	8′	—		54		—		1.41	
24–35		—		52	5	—		1.41	0.89

[1] Subject and diet numbers correspond with those in Southern Cooperative Series Bulletin no. 64 ('59).

[2] Standard deviation of the observations.

TABLE 5

Values for urinary folic acid excretion reported in the literature and from these studies

Study	Subjects	Urinary folic acid[1]
		μg/day
Cheldelin, '42	15 normal subjects	10.8
Denko et al., '46a	7 young men	3.99
Denko et al., '46b	5 young men	3.00
Johnson et al., '45	4 adult males	8
Odland and Parsons, '50	29 women	3.7
Steinkamp et al., '46	9 normal subjects	2.32
These studies, '54	11 girls, 7–9 years	1.24
'56	12 girls, 7–9 years	1.13
'58	12 girls, 7–9 years	1.41

[1] *S. faecalis* used as test organism.

Only a "trace" of folic acid was excreted in one or more metabolic periods by three girls in 1954, two in 1956, and 5 in 1958. These low excretions, which have been excluded from the averages in this report, usually occurred when a subject was ill and was treated with antibiotics. The details of any medication used are given in the Southern Cooperative Series Bulletin no. 64 ('59).

SUMMARY

Pantothenic acid and folic acid content of food and urine was determined for 35 girls, 7 to 9 years of age, maintained with controlled diets. Three series of metabolic studies were made.

Pantothenic acid intakes in the three studies averaged 4.49, 5.00, and 2.79 mg per child per day; urinary excretions averaged 2.85, 1.71, and 1.31 mg per day, respectively. In general, with the higher intakes of pantothenic acid the urinary excretion tended to be higher.

With an increased protein intake there was an increased pantothenic acid intake since many of the foods high in pantothenic acid are likely to contain considerable protein.

Pantothenic acid excretion for the girls in these studies could not be related to body size.

Folic acid intake in the three studies averaged 98, 80, and 52 μg per child per day; urinary excretion averaged 1.24, 1.13, and 1.41 μg per day, respectively.

Excretion of folic acid in the urine accounted for less than 3% of the intake. The amount excreted did not show relationship to folic acid intake, pantothenic acid intake, protein intake, or body weight.

Diets, made up of ordinary foods planned to meet the National Research Council's recommended allowances of well known nutrients for children 7 to 9 years of age, supplied from 4 to 5 mg of pantothenic acid and from 80 to 100 μg of folic acid per day.

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Gingivitis-Ascorbic Acid Deficiency in the Navajo

I. ASCORBIC ACID IN WHITE CELL-PLATELET FRACTION OF BLOOD[1,2]

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The dental officers at the Intermountain School, Brigham City, Utah, observed hemorrhagic gingivitis in approximately 10% of the Indian children arriving at the school. Preliminary administration of large doses of ascorbic acid in several instances resulted in dramatic improvement of the inflamed gums. Plans were then made to study the relationship between the ascorbic acid nutriture of Navajo subjects with and without the inflamed gum condition, hemorrhagic hyperplastic gingivitis.[4]

A detailed study of the dietary background and nutriture of Navajo Indians on their reservation was reported in 1956 by Darby et al. Gingival lesions were observed in many of the subjects examined. Serum ascorbic acid levels were low in the majority of cases. At least half of all groups had ascorbic acid levels below 0.3 mg per 100 ml of serum. A positive correlation between serum levels and the incidence of gingival lesions, however, was not found. Poor oral hygiene and inadequate dental care were felt to be the major factors in the high prevalence of gingival disorders.

In a study of school children with sore gums or gingivitis in New Mexico, Campbell et al. ('53) found that the very low intake of dietary vitamin C was reflected in low levels of vitamin C in the blood. When half the children were given a vitamin C supplement, serum values returned to normal and the gingivitis responded satisfactorily.

The effect of ascorbic acid supplementation or of the regular diet on the ascorbic acid content of the white cells and platelets of a group of Navajo students with hemorrhagic hyperplastic gingivitis is reported. The ascorbic acid content of the white cells and platelets was chosen for study because the concentration of ascorbic acid in this fraction of the blood has been shown to be a good measure of the ascorbic acid nutriture of subjects, particularly at low levels of ascorbic acid intake (Lowry, '52; Morse et al., '56; and others).

Papers on the other two phases of this study, clinical findings on the atypical gingivitis and dietary intakes, will appear later (Glauser and Humphreys,[5] McDonald).[6] Pertinent findings may be summarized as follows.

Gum conditions of the paired gingivitis groups were similar at the initial examination. At the end of three weeks, the gum conditions in both groups had improved, but the extent of improvement in the group receiving 300 mg of ascorbic acid supplement daily was markedly greater. Six months later the gum conditions in the two groups were almost identical. Results indicate that the daily ascorbic acid supplement of the diet contributed to the earlier response and healing of the hemorrhagic hyperplastic gingivitis.

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[2] Approved as Utah Agricultural Experiment Station Journal Paper no. 165.

[3] Present address: Cleveland, Ohio.

[4] Glauser, R. O., and P. K. Humphreys. Gingivitis-ascorbic acid deficiency in the Navajo. II. Dental aspects. Manuscript in preparation.

[5] See footnote 4.

[6] McDonald, B. S. Gingivitis-ascorbic acid deficiency in the Navajo. III. Dietary aspects. Manuscript in preparation.

The mean dietary intake for the boys during the three-week test period was 78 mg of ascorbic acid and for the girls, 62 mg. Seventeen per cent of the subjects had food intakes which met their NRC ('53) recommended allowances for ascorbic acid. Twenty-four per cent consumed less than two-thirds of their NRC allowance. More subjects with adequate intakes were in the 10 to 12-year group than in the older age group. Likewise more boys met their NRC allowance than girls.

METHOD OF PROCEDURE

The 283 Navajo subjects in this study were selected by the dental officers of the school health center from all students enrolling at the Intermountain School. Selection was made on the basis of the oral condition of the gums. The experimental subjects (186) had atypical bright red gingivitis or hemorrhagic hyperplastic gingivitis. The control subjects (97), of comparable age and sex, had normal gingiva. The division of subjects into groups for ascorbic acid supplementation (300 mg daily in tablet form for 3 weeks) is shown in table 1. The majority of subjects were from 12 to 18 years of age. All sections of the Navajo reservation and several nearby communities were represented.

The students arrived at the Intermountain School during a 6-day period. Each student was given a medical and dental examination on the day of arrival. During this week, menus served by the cafeteria were adjusted to supply approximately 50 mg of ascorbic acid daily.

Venous blood samples were taken for analyses for all subjects at the beginning of the study which was one week after arrival of the first students, three weeks later during which time groups 1 and 3 had been receiving 300 mg of ascorbic acid daily in tablet form in divided doses, and 6 months after the initiation of the study. At the end of the 6th week blood samples were taken for analysis from the subjects in group 2 following their ascorbic acid supplementation period (see table 1). The white blood cells were isolated immediately, trichloroacetic acid was added, and the samples frozen for later analysis for ascorbic acid by the method of Bessey and co-workers (György, '50).

RESULTS

No sex difference was noted for white cell-platelet ascorbic acid content. Initial mean white cell ascorbic acid values were significantly lower ($P < 0.01$) for subjects who had gingivitis than the values for those of the control groups (table 2). According to the value of 15 mg or more per 100 gm of white cells which Crandon et al. ('58) reported for normal subjects, the control groups had normal ascorbic acid white cell values, whereas values of the gingivitis groups fell within the subnormal classification. If Lowry's ('52) figure of 25 mg per 100 gm of white cells was used as the average concentration for normal subjects, all groups of Indian subjects had mean white cell ascorbic acid values well below normal.

The use of 300 mg of ascorbic acid daily for three weeks as a dietary supplement resulted in significant increases ($P < 0.01$) in ascorbic acid content of the white cells. The response of subjects in group 1 of those having gingivitis and of group 3 from the controls was similar (25 and 25.6 mg).

Even higher white cell ascorbic acid values were shown by subjects in group

TABLE 1

Ascorbic acid supplementation by groups, number, and age of subjects

Gum condition	Group no.	Ascorbic acid supplement			No. of subjects			Age	
		1 to 3 weeks	4 to 6 weeks	7 to 20 weeks	Boys	Girls	Total	Boys	Girls
		mg/day	*mg/day*	*mg/day*					
Atypical gingivitis	1	300	300	—	38	55	93	12–20	11–22
	2	placebo	—	—	37	56	93	12–20	12–20
Normal gingiva	3	300	—	—	23	24	47	12–19	12–18
	4	placebo	—	—	24	26	50	12–19	12–21

TABLE 2

Ascorbic acid content of white cells and platelets of Indian subjects with and without gingivitis

Gum condition	Group no.	300-mg ascorbic acid supplement	Analyses[1]			
			Initial	At 3 weeks	At 6 weeks	Final
		weeks of supplementation	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
Atypical gingivitis	1	1–3	14.0±6.7	25.0±7.8	—	26.7±7.7
	2	4–6	12.2±5.1	17.2±5.3	32.9±5.8	25.0±7.6
Normal gingiva	3	1–3	17.2±6.4	25.6±7.2	—	27.4±6.8
	4	—	15.9±6.6	17.9±6.7	—	23.2±8.8

[1] Mean ± standard deviation.

2 at the end of the 6th week. These values for group 2 were approximately one-third greater than those for group 1 whose supplement period was the first three weeks of the study (32.9 vs. 25 mg). These high levels of group 2, however, were not maintained with the cafeteria diet.

After 6 months at the Intermountain School during which time the students ate at the school cafeteria, differences between groups in white cell ascorbic acid values had disappeared (25.2 and 25.9 mg). Differences between initial and final values were highly significant for both groups of subjects. The initial ascorbic acid values for the gingivitis group almost doubled while those for the control group increased by one-half.

Crandon et al. ('58) classified subjects according to their white cell-platelet ascorbic acid as follows: saturated, 30 mg or more per 100 gm white cells; normal, 15 mg or more; subnormal, greater than 8 mg; deficient, less than 8 mg; and scorbutic, less than 4 mg. In figure 1 is shown the percentage of subjects in this study which fell within each of these divisions for the initial and final analyses. The percentage of subjects in the gingivitis groups who had white cell ascorbic acid initial values below the normal value of 15 mg was approximately double that found in the control groups. More subjects with gingivitis had values that were within the deficient and scorbutic levels than did the controls.

After three weeks of supplementation with 300 mg of ascorbic acid, groups 1 and 3 had few subjects with values below 15 mg per 100 gm of white cells. The percentage having values in the range of 15 to 25 mg remained the same. The ratings above 25 mg showed great gains. Group 1 had 26% at the saturation level and 31% with values from 25 to 30 mg, whereas the controls in group 3 had 38 and 15%, respectively, for these two classifications. Groups 2 and 4 which had not received the suppplement showed approximately the same ratings as at the time of the initial analyses. Three weeks later, after group 2 had received 300 mg of ascorbic acid daily, 71% had values at the saturation level and 25% with values between 25 and 30 mg.

At the time of the final analyses no subjects rated scorbutic and only one subject in group 1 was deficient. Only a few in each group rated subnormal. The majority of subjects in all groups had normal values of 15 mg or above with one-fourth to one-third of the subjects in all groups rating within the saturated levels.

COMMENTS

The low initial white cell ascorbic acid values of the subjects in this study were similar to those found by Steele et al. ('55) following 38 to 42 days of diets containing 10 mg or less of ascorbic acid. Their subjects were then given 20, 30, and 40 mg of ascorbic acid daily for periods of 14 or 11 days. Only the 40-mg supplement resulted in a significant increase in white cell ascorbic acid values.

Initial white cell ascorbic acid levels in this study might have been still lower if analysis had been made the first day the subjects came from the reservation. Some subjects ate the cafeteria diet containing 50 mg of ascorbic acid daily for one to 7 days. The response of subjects in groups

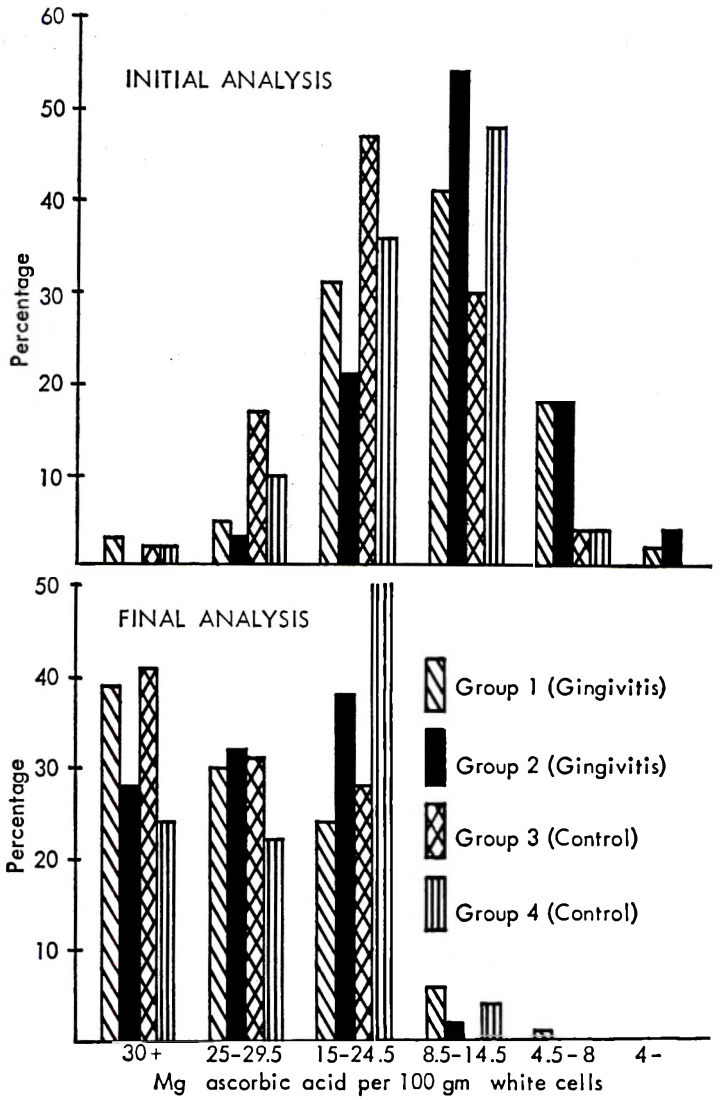

Fig. 1 Percentage of Indian students by groups according to white cell-platelet ascorbic acid content.

2 and 4 during the first three weeks, however, did not indicate that rapid changes were taking place within a few days. The control boys and girls in group 4 receiving calculated daily dietary intakes of 75 and 62 mg of ascorbic acid[7] showed increases of 9 and 15% in white cell ascorbic acid levels during the three weeks. Larger increases (40%) were shown by the boys and girls in group 2, with similar dietary intakes of 80 and 63 mg of ascorbic acid. The white cell ascorbic acid levels were then normal with a range of 17 to 19 mg.

When the level of white cell ascorbic acid of the subjects before supplementation was considered in relation to the percentage increase in ascorbic acid content during supplementation, the response of the subjects in the gingivitis and control groups was not the same (78 vs. 49% increase). An even higher increase (91%)

[7] See footnote 6, page 352.

was shown by the group 2 subjects even though their white cell values were higher when the supplementation was started. The work of Steele et al. ('55) has indicated that the nutriture of the subject may have some influence on the response to supplementation of the white cell ascorbic acid level. Subjects of Lamden et al. ('60) with higher initial values (29 mg) showed only a 27% gain in white cell ascorbic acid content after taking a supplement of approximately 300 mg of ascorbic acid daily for two-and-a-half years. They found no significant correlations between ascorbic acid intake and white cell ascorbic acid level.

SUMMARY

Ascorbic acid levels in white cells of Navajo Indian students (283) with and without hemorrhagic hyperplastic gingivitis were determined within a week after arrival at the Intermountain School, after supplementation with 300 mg of ascorbic acid, and at the end of 6 months. Data from this study show the following:

1. Initial mean white cell ascorbic acid values were significantly lower for subjects who had gingivitis than the values for the control groups.

2. Administration of 300 mg of ascorbic acid daily for three weeks resulted in significant increases in ascorbic acid levels. Most all of the subjects had normal values with 26 to 71% having values indicating saturation. These high levels could not be maintained with the cafeteria diet.

3. The percentage increase in white cell ascorbic acid content during the supplementation period was lowest for the control subjects (49%) and highest for the gingivitis group receiving the cafeteria diet for three weeks followed by a daily supplement of 300 mg of ascorbic acid for three weeks (91%). The other gingivitis subjects who received their supplement the first three weeks showed an increase of 78%.

4. Six months after initiation of the study all groups of subjects had normal values that were similar.

ACKNOWLEDGMENTS

The authors wish to extend grateful acknowledgment to Dr. R. O. Glauser, Dr. P. K. Humphreys, Dr. Toyo Shimizu, and Mrs. B. S. McDonald for valuable assistance in carrying out this study; to W. P. Bethuy for taking the blood samples; to the subjects; and to D. Sissons for the statistical analyses.

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Glyoxalase Activity in Liver and Blood of Thiamine-Deficient Rats[1]

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From time to time various investigators have reported that tissues of thiamine-deficient animals show decreased ability to convert pyruvaldehyde (methylglyoxal) to lactic acid. The conversion of pyruvaldehyde to lactate involves a two-step enzymatic process with glutathione (GSH) as cofactor (Racker, '51; Crook and Law, '52). In the first reaction, catalyzed by glyoxalase I, pyruvaldehyde condenses with GSH to form a thioester intermediate, S-lactoyl-glutathione. The thioester is then hydrolyzed by a second enzyme, glyoxalase II, to lactate and free GSH.

In the early literature Findlay ('21) reported that glyoxalase activity of liver extracts from thiamine-deficient pigeons was reduced by about 50%. Vogt-Møller ('31) demonstrated the formation of pyruvaldehyde by livers of thiamine-deficient animals when hexose diphosphate was used as substrate. Normally pyruvaldehyde cannot be demonstrated in tissue extracts because of the high activity of the glyoxalase system. Johnson ('36) could not identify pyruvaldehyde in the tissues of polyneuritic pigeons. Much more recently, Salem ('54) has described experiments which indicate that livers from thiamine-deficient rats are almost completely incapable of converting pyruvaldehyde to lactate. The conversion was restored to normal by feeding thiamine. He also reported the presence of pyruvaldehyde in the urine of thiamine-deficient rats and showed, in addition, that pyruvaldehyde was produced by liver suspensions from the deficient animals when incubated for long periods with hexosediphosphate. Inasmuch as pyruvaldehyde is a toxic substance, the above author has reiterated the suggestion of Vogt-Møller in 1931 that the symptoms of thiamine deficiency may, in part, be those of pyruvaldehyde toxicity.

The failure of thiamine-deficient liver to effect the glyoxalase reaction could be due to the disappearance of either or both of the enzymes glyoxalase I and glyoxalase II or to a lack of the intermediate GSH. Salem ('54) was unable to conclude which of these factors was absent. Because of this, and in view of the suggestion that pyruvaldehyde toxemia may be a factor in thiamine deficiency, it seemed worthwhile to examine this metabolic system more closely. Pyruvaldehyde is catabolized vigorously by red cells (Klebanoff, '56; Collier, '53) and its accumulation in the urine would likely necessitate the disappearance of one or more of these factors from the blood. This report deals with some of our findings concerning glyoxalase activity in the liver and blood of thiamine-deficient rats.

METHODS

Eighteen male weanling rats (Wistar strain, mean weight 48 gm) were placed in metabolism cages and fed a vitamin B-complex test diet[2] nutritionally adequate in all respects except thiamine. Twelve control animals (mean weight 49 gm) received an identical ration to which had been added thiamine hydrochloride (2 mg per 100 gm of diet). Animals were sacrificed in the terminal stages of the deficiency (at approximately 4 weeks) when there was complete loss of appetite, marked loss of weight, difficulty in use of the limbs and convulsions when aroused.

Preparation of extracts

The deficient animals were sacrificed in groups of three and the tissues pooled.

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[1] This work was supported by a grant from the National Research Council of Canada.
[2] Nutritional Biochemicals Corporation, Cleveland.

Normal animals, because of their larger size, were used individually.

Blood. Three deficient animals were stunned and after severing the neck vessels, the blood was collected in a graduated tube containing heparin. For glyoxalase determinations an aliquot (0.5 to 1.0 ml) was laked with 5 volumes of water and placed in ice. Another 1.0-ml aliquot was homogenized in a Potter-Elvehjem homogenizer (Teflon pestle) for one minute with 0.2 ml of 25% sulfosalicylic acid at 0°, followed by a further three-minute homogenization after the addition of 2.0 ml of 2% sulfosalicylic acid. The extract was placed in ice until the liver extracts were prepared.

Liver. After collecting the blood, the abdomens were opened and blood was removed from the livers by perfusion with saline (15 ml per animal) through the inferior vena cava and the portal vein. The livers were then removed, blotted, weighed and chilled in ice. For glyoxalase assays, one-gram portions of each of the three livers were combined and homogenized in a Potter-Elvehjem homogenizer with 15 ml of 0.15M KCl for three minutes at 0°. For GSH determinations, additional one-gram portions of each liver were combined and homogenized with 3 ml of 5% sulfosalicylic acid for one minute with a further three-minute homogenization after the addition of 12 ml of 2% sulfosalicylic acid. In the normal series, a liver homogenate and blood hemolysate were similarly prepared from each individual animal. Normal livers were perfused with 40 ml of saline and a 3-gm portion of the liver used to prepare the KCl extract and an additional 3 gm to prepare the sulfosalicylic acid extract. The preparations were immediately centrifuged at 20,000 × G for 20 minutes at 0°C. The laked blood and KCl homogenates were used at once for glyoxalase assays. The sulfosalicylic acid supernatants were kept at − 18° until assayed for GSH (not over 4 hours).

Assays

Glyoxalase was determined by the manometric method of Hopkins and Morgan ('45), which depends on the rate of evolution of CO_2 from lactate formed in the presence of bicarbonate. Glutathione was determined by the manometric glyoxalase method (Patterson and Lazarow, '55) using yeast acetone powder as the enzyme. Pyruvaldehyde was estimated in urine manometrically by the procedure of Salem ('54) and by the glyoxalase I test system of Racker ('51). Chromatography of urine concentrates was carried out in butanol-ethanol-water (40:50:10, v/v) (Salem, '55). Spectrophotometric glyoxalase I and glyoxalase II assays were conducted by the method of Racker ('51), except that the buffer used was Tris (hydroxymethyl) aminomethane (Tris-HCl), pH 7.5. S-Lactoyl-glutathione was prepared enzymatically using highly purified yeast glyoxalase I (Racker, '51). Protein was determined by the method of Lowry et al. ('51).

RESULTS

Preliminary. Under the conditions of the assay, CO_2 evolution was linear with respect to time throughout the experimental period, and for much longer periods. In figure 1 is shown a typical assay of a liver homogenate and blood hemolysate from a normal rat. It can be seen that GSH greatly increases the rate of reaction in both liver and blood. GSH is thus the rate-determining factor in these extracts. This does not mean, however, that the thiol is necessarily rate-determining in the cell. The effect noted here may be the result of dilution during preparation of the extracts. The concentration of GSH used throughout (2 μmoles) was sufficient to saturate the enzyme system.

In contrast with the enzyme from yeast, activity in liver varied somewhat with bicarbonate concentration. This necessitated careful neutralization of all solutions before use. The concentration of bicarbonate chosen for routine use (40 μmoles) gave nearly maximal rates and prevented shifts in pH during the experimental interval. Increasing the amount of bicarbonate added to 60 and 100 μmoles, decreased the rate by 14 and 26%, respectively. Enzyme activity from blood was independent of bicarbonate concentration in agreement with Klebanoff ('56).

Changes during thiamine deficiency. Liver and blood glyoxalase activities of normal and thiamine-deficient rats are shown in table 1. Glyoxalase activity is

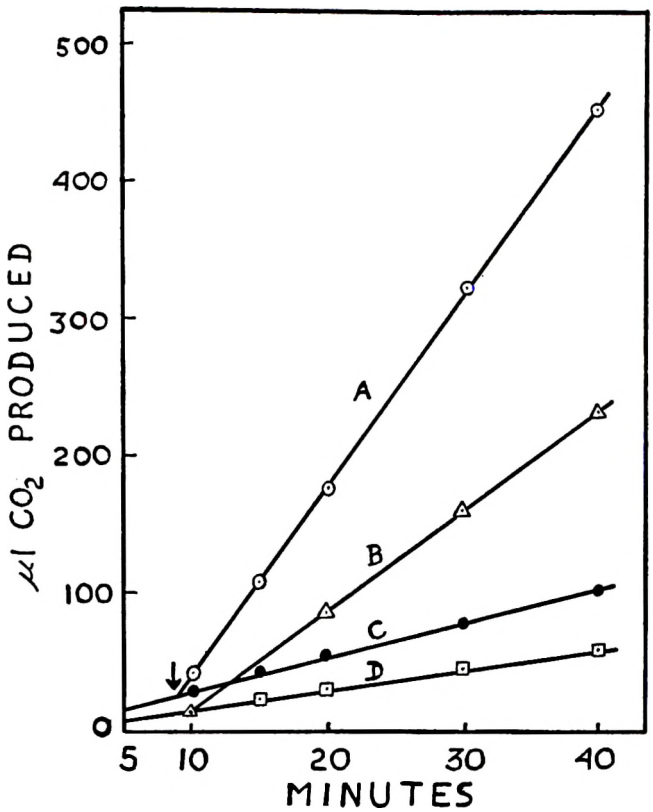

Fig. 1 Rat liver and blood glyoxalase assay. To chilled Warburg flasks was added $NaHCO_3$, 40 μmoles; pyruvaldehyde, 100 μmoles; and water to a final volume of 3.0 ml. Glutathione, 2.0 μmoles, was placed in the side-arm of appropriate flasks. Liver extract (0.2 ml) and blood hemolysate (0.3 ml) prepared as described in the text, were added to appropriate flasks and the system was gassed with 95% N_2–5% CO_2. After closing the stopcocks CO_2 evolution was recorded at 10-minute intervals for 40 minutes. The temperature was 30°. GSH was added at the arrow. A, liver with GSH added; B, blood with GSH added; C, liver, no GSH; D, blood, no GSH. In the absence of pyruvaldehyde, CO_2 evolution never exceeded 8 μl during the experimental interval.

TABLE 1

Liver and blood glyoxalase activities of normal and thiamine-deficient rats[1,2]

	No. of animals	Liver		Blood	
		Glutathione	No glutathione	Glutathione	No glutathione
Normal	12	380 ± 15.0[3]	100 ± 8.0	230 ± 9.2	45 ± 3.0
Thiamine-deficient	18	211 ± 13.1	40 ± 5.0	224 ± 4.8	28 ± 1.3
		($P < 0.001$)[4]	($P < 0.001$)		($P < 0.001$)

[1] Conditions are as described for figure 1.

[2] Results are expressed as μl CO_2 produced during the interval 10 to 40 minutes by 0.2 ml of liver homogenate and by 0.3 ml of blood hemolysate.

[3] Values are the mean ± S.E.

[4] P values refer to significance of differences from normal.

lowered in homogenates of the deficient livers when assayed with and without GSH. The decrease, however, is not nearly as great as that reported by Salem ('54). The results reported here are based on volume of homogenate. Protein content of the homogenates of deficient liver did not differ greatly from normal liver (table 3).

Liver GSH is also somewhat lowered in the thiamine-deficient animals (table 2). Presumably, this could account, in part, for decreased glyoxalase activty in the absence of the thiol. Lowered activity in the presence of saturating amounts of GSH, however, indicates there must be a lowered enzyme activity in the deficient livers as well.

Thiamine-deficient blood shows no decreased glyoxalase activity when assayed with GSH. There is decreased activity, however, in the absence of the thiol (table 1). Blood GSH is markedly reduced in the thiamine-deficient animals (table 2). The results indicate that decreased blood glyoxalase activity may be due alone to reduced GSH levels.

It seemed important to know whether lowered glyoxalase activity in deficient liver was due to decreased glyoxalase I or glyoxalase II. Separate optical assay for glyoxalase I by the procedure of Racker ('51) is difficult in normal liver and blood because this enzyme is rate limiting. Therefore, in the assay no optical changes occur because the thioester intermediate, S-lactoyl-glutathione, is hydrolyzed by glyoxalase II as rapidly as it is formed. Glyoxalase II, however, can be assayed optically in the presence of glyoxalase I using S-lactoyl-glutathione as substrate. A significant reduction in glyoxalase II activity occurred in the livers of the deficient rats as shown in table 3. When liver homogenates of the deficient animals were assayed in the glyoxalase I system no optical changes occurred, however, indicating that glyoxalase I was still rate limiting despite a 45% reduction in glyoxalase II. From these results it would appear that lowered glyoxalase II levels in the deficient livers would not account for decreased overall glyoxalase activity and this must result from reduced glyoxalase I activity. In

TABLE 2

Liver and blood glutathione of normal and thiamine-deficient rats[1]

	No. of animals	Liver	Blood
		mg/100 gm	*mg/100 ml*
Normal	8	129 ± 9.2[2]	36 ± 3.5
Thiamine-deficient	12	97 ± 3.0 ($P < 0.01$)[3]	12 ± 1.6 ($P < 0.001$)

[1] Extracts were prepared and assayed as described in the text.
[2] Values refer to the mean ± S.E.
[3] P values refer to significance of differences from normal.

TABLE 3

Glyoxalase II activity of rat liver and blood[1]

	Liver homogenate		Blood hemolysate	
	Normal	Thiamine-deficient	Normal	Thiamine-deficient
Number of animals	7	18	6	18
Protein, mg/ml	16.4 ± 0.32[2]	14.6 ± 0.40	42.2 ± 1.6	44.3 ± 1.3
Glyoxalase II (μmole substrate hydrolyzed/min./mg)	0.75 ± 0.02	0.41 ± 0.01	0.020	0.021

[1] The homogenates and hemolysates are described in the text. Reaction velocities were determined by following the initial rate of disappearance of absorbance at $\lambda = 240$ mμ due to hydrolysis of S-lactoyl-glutathione (Racker, '51). The molecular extinction coefficient of S-lactoyl-glutathione at this wavelength was taken as 3300.
[2] Values are the mean ± S.E.

table 3 it is also shown that glyoxalase II activity in thiamine-deficient blood is not different from that in normal blood.

Failure to demonstrate pyruvaldehyde in thiamine-deficient urine. Three methods were used to determine whether pyruvaldehyde was present in the urine of the deficient animals. By the chromatographic procedure using butanol:ethanol:water (40:50:10, v/v), one can detect 0.02 μmole of the compound after spraying with ammoniacal silver nitrate. The glyoxalase I system is less sensitive but 0.2 μmole can easily be detected. The manometric method (Salem, '54) is capable of detecting approximately 1 μmole quantities. Known amounts of the aldehyde, when added to urine, can be quantitatively accounted for by the two latter methods. In contrast with the results of Salem ('54), repeated attempts using the above three methods failed to produce any evidence for the presence of pyruvaldehyde in the urine of the deficient animals at any time during the deficiency.

DISCUSSION

The results reported here are not entirely in agreement with those of Salem ('54, '55). Liver glyoxalase activity is significantly lowered in the thiamine-deficient animals, but not nearly as markedly as reported by the latter author, and pyruvaldehyde was not found in the urine. The reason for this is not clear. The animals used in these experiments were severely deficient and in almost every case were only several hours from death. Of possible significance is that the reaction curves reported by Salem are not linear, and in the deficient animals the reaction appears to terminate completely after 10 minutes. This would indicate that some component had become limiting or that the enzyme has lost activity. In our experiments all reaction rates were consistently linear well beyond the experimental interval, indicating there was no loss of enzyme activity during incubation in either the normal or deficient series.

Because substantial levels of glyoxalase remained in liver (40% as assayed without GSH) and in blood (62% assayed similarly) it was not unexpected that pyruvaldehyde could not be found in the urine. The production of the aldehyde from hexose diphosphate appears to be a relatively slow process compared with the high activity of the glyoxalase system. It is posssible to calculate from Salem's data ('54) that 0.5 gm of liver pulp (pretreated with kidney antiglyoxalase to destroy GSH) is capable of forming only about 20 μmoles of pyruvaldehyde in 24 hours at 37°. Assuming that the assay for glyoxalase in the absence of added GSH more nearly approximates conditions in the intact cell, it can be calculated from our data that 0.5 gm of liver from the thiamine-deficient animals could still metabolize 2400 μmoles of pyruvaldehyde during the same period. Thus, although glyoxalase activity in both liver and blood is lowered in the deficient animals, this decrease is not adequate to permit accumulation of the aldehyde.

It appears that the lowered glyoxalase activity of thiamine-deficient blood can be explained simply on the basis of lowered GSH. This is consistent with the finding that when assayed with added GSH, activity is equivalent to the normal. The situation in liver seems more complex and it appears that glyoxalase I, glyoxalase II and GSH might all be lowered in the deficiency. By the methods used here it is not apparent which of these factors is rate-determining in the intact cell, but endogenous GSH is clearly rate-limiting in homogenates.

It should be emphasized that lowered GSH and glyoxalase levels may not be a direct result of a deficiency of the vitamin. Rather they may be a reflection of secondary factors imposed by a severely reduced food intake. All animals had suffered severe loss of weight and, at the time of sacrifice, food consumption had virtually ceased. Leaf and Neuberger ('47) have established that a diet low in protein causes a marked reduction in liver GSH. This decrease is apparently not accompanied by changes in blood thiol, however (Edwards and Westerfeld, '52). Whatever the ultimate cause, significant glyoxalase activity still remains in these severely deficient animals. It seems reasonable to conclude that pyruvaldehyde metabolism is not an important factor in thiamine deficiency.

SUMMARY

Pyruvaldehyde catabolism has been examined in liver and blood of normal and thiamine-deficient rats. Liver glyoxalase activity was reduced in thiamine-deficient animals and this was due to lowered glyoxalase I levels and probably decreased glutathione. Blood glyoxalase activity was lowered in thiamine deficiency and this appears to result from lowered glutathione levels alone. It is not known whether these changes are a direct result of a deficiency of the vitamin or a consequence of severely reduced food intake. Pyruvaldehyde could not be detected in the urine of deficient animals. The data presented are consistent with the idea that pyruvaldehyde metabolism is not an important factor in thiamine deficiency.

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Chemical and Histological Changes in the Femurs of Chicks Fed Lysine-Deficient Diets[1]

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Lysine deficiency has been known to result in negative nitrogen balance in man (Albanese et al., '41); in loss of weight (Kligler and Krehl, '50, '52), retarded epiphyseal growth (Haggar et al., '55), decreased hematocrit and hemoglobin values (Gillespie et al., '45) in the rat and retarded growth and changes in the feather coloring in the growing chick (Patrick, '53; Klain et al., '57). Bavetta et al. ('54, '55) reported pathological changes in the teeth and bones of rats due to lysine or tryptophan deficiency and Fischer ('48) found that lysine deficiency resulted in diminished growth of osteoclasts in tissue cultures. Rohdenburg ('58) studied the effect of lysine deficiency on the chemical composition of the tibias of albino rats finding an increase in fat and a decrease in the water content of the bones; no further studies were carried out to determine the cause of these findings.

The present study was conducted to determine the effect of lysine deficiency on the chemical and histological composition of the long bones of New Hampshire chicks.

MATERIALS AND METHODS

One-day-old New Hampshire male chicks were distributed by weight among the experimental groups of each trial. They were fed a basal ration containing the following per 100 gm: sesame oil meal, 40.0; ground yellow corn, 56.7; minerals,[2] 3.0; cod liver oil, 0.3; and 5 ml of a vitamin solution containing per milliliter: inositol, 10; vitamin K, 5; choline chloride, 160; *p*-aminobenzoic acid, 1; niacin, 10; riboflavin, 2; pyridoxine·HCl, 2; thiamine·HCl, 2; Ca pantothenate, 6; biotin, 0.04; folic acid, 0.2; and vitamin B_{12}, 0.003 mg. This basal ration, containing 20% of crude protein, was calculated to contribute 66% of the total lysine requirement for the chick at the 20% protein level (Grau, '48). In the experimental rations L-lysine hydrochloride was added in place of an equivalent weight of corn bringing the total lysine content of the diet to 74, 83 and 100% of the suggested requirement.[3] The birds were kept in cages with raised screen bottoms and feed and water were supplied ad libitum. The various experiments are described under Results.

The birds were weighed every week and 4 or 5 from each experimental group were taken at weekly intervals, bled by heart puncture for hemoglobin and hematocrit determinations, sacrificed and both femurs dissected, cleaned, weighed and their length determined with a caliper. In some experiments the livers were also removed, weighed and prepared for chemical analysis.

One of the femurs was fixed in 10% formaldehyde for histological studies and the other was used for chemical analysis. For moisture determinations, the bones were dried in an oven at 100°C for 18 hours, cooled and weighed. Fat was determined by first boiling the dry bones in 95% ethyl alcohol under reflux for 24 hours and then extracting in a Soxhlet extractor with ethyl ether for the same period of time. After drying they were again weighed and the fat content calculated by difference. The bones were then ashed at 500°C for 12 hours and the calcium and phosphorus content in the ash determined, using the

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[1] INCAP Publication I-193.

[2] In per cent: Ca carbonate, 1.0; bone meal, 1.0; salt, 1.0; and trace elements.

[3] The authors are indebted to E. I. duPont de Nemours and Company, Inc., Wilmington, Delaware, for supplying the L-lysine hydrochloride, and to Mead Johnson and Company, Evansville, Indiana, for the cod liver oil used in this study.

standard AOAC ('50) methods. In one experiment protein was determined by the macroKjeldahl procedure using selenous acid as the catalyst. All data were calculated on a fresh basis. When liver fat was determined, the vacuum-oven dried, homogenized tissue was extracted with ethyl ether in a continuous extracting apparatus for 6 hours. Hemoglobin was determined in whole blood by the cyanmethemoglobin method (Cannan, '58), and hematocrit by the micro method of McGovern et al. ('55).

For the histological studies, the bone previously decalcified in an ionic bone decalcifier, was sectioned in three parts: a cross section at the middle part of the diaphysis and two longitudinal frontal sections passing through each of the epiphyseal cartilage plates at the level of the zone of endochondral ossification of the femur. Histological sections cut at 6 mμ were stained with hematoxylin-eosin and phosphotungstic acid hematoxylin (PTA Mallory stain). In addition to routine observations the following measurements were made on the histological sections: (1) percentage of myeloid tissue in the bone marrow; (2) thickness of the compact bone at the middle part of the diaphysis; and (3) thickness of the area of endochondral ossification.

For the determination of the percentage of the myeloid tissue, the cross section at the middle part of the diaphysis was used. A microscopic grid, containing 100 squares, was used and the percentage of fat and myeloid tissue was determined in 10 areas with a total count of one-thousand squares. The thickness of the cortical bone at the middle of the diaphysis was measured microscopically at 4 opposite sites (3, 6, 9, and 12, clockwise) and the average calculated. The thickness of the endochondral ossification areas was also microscopically measured at 6 points, three on each side of the middle line and the average calculated. Special care was taken to measure the cartilage plate from the area where the hyaline cartilage starts to undergo degenerative changes (hypertrophic cartilage cells and vacuolization) to the beginning of the spicule formation.

RESULTS

Experiment 1. One-day-old New Hampshire chicks were fed a commercial concentrate containing 20% of crude protein. The fat content of the livers and femurs was determined weekly in 5 birds from each group. This experiment was carried out to give an idea of the normal fat content in the two tissues studied. The bone and liver fat data of normal birds from hatching time to 9 weeks of age are shown in figure 1. The percentage of liver fat decreased rapidly after the first day of age and remained fairly constant through-

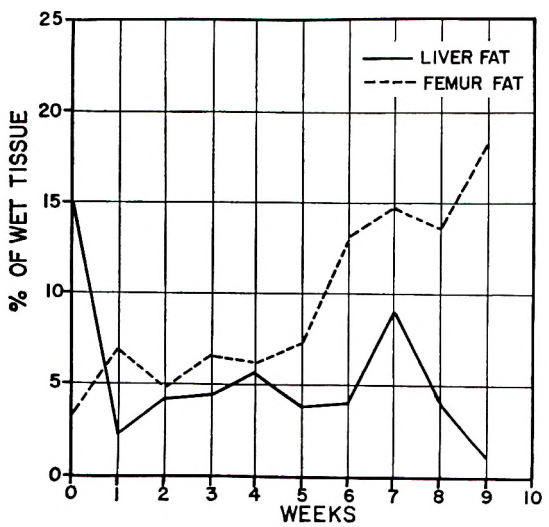

Fig. 1 Femur and liver fat of New Hampshire chicks.

out the experimental period; bone fat increased steadily with increasing age.

Experiment 2. Two groups of chicks were used; one group was fed the control diet with 66% of the lysine requirement, and the other group received the same ration supplemented with L-lysine hydrochloride to provide the full amino acid requirement for the chick. Liver fat was not affected by the lysine deficiency, but bone fat increased and the water content decreased significantly as shown in table 1. These figures represent the average of 5 chicks from each group.

Experiment 3. In order to determine whether other constituents of the bone changed as a result of the lysine deficiency, two experiments were carried out using experimental rations containing 66, 74, 83, and 100% of the lysine requirement. In figure 2 are shown the combined data for body weight in experiments 3 and 4 at the different levels of lysine fed; better weights were obtained with increasing levels of lysine.

In experiment 3 one femur from each of the 4 chicks sampled per group was used for histological studies and the other for chemical analysis of water, fat and ash content. Calcium and phosphorus content of the ash was also determined but the measurement of protein was not feasible. As shown in table 2, the absolute weight of the femur and its length increased with increasing levels of lysine. When the weight of the femur was expressed as percentage of body weight there was no change. This was expected, since the body weight decreased with decreasing levels of lysine. Chemical analysis showed that, as lysine content of the ration increased, bone moisture content also increased, but bone fat decreased. The differences were statistically significant at the 1% level. The percentage of ash and its calcium and phosphorus content remained constant under the different treatments.

Experiment 4. Growth data from this experiment have already been mentioned. In addition, ash content was determined in the right femur, protein in the left femur and moisture and fat in both bones from each of the 4 chicks sampled per group. Data in table 3 show the same inverse relationship between moisture and fat content which was observed in experiment 3. Ash content did not change significantly but the protein content was lower ($P < 0.05$) in the bones of chicks fed low levels of lysine. Hemoglobin and hematocrit values (table 4) also tended to correlate with the lysine level of the ration. As the lysine in the ration increased, there was a corresponding rise in hematocrit and hemoglobin values. These changes were statistically significant at the 1% level for hemoglobin and the 5% level for hematocrit.

Histological findings. The percentage of myeloid tissue increased ($P < 0.01$) with the increase of lysine in the ration, this difference being more striking during the second and third weeks (table 5, column 3).

A microphotograph of the cross section of the middle of the diaphysis in 4 chicks (one from each of the 4 experimental groups during the third week of treatment) indicates that the amount of myeloid tissue increased and fat decreased with higher levels of lysine (figs. 4–7). These findings are consistent with the

TABLE 1

Liver and femur fat in the New Hampshire chicks fed two levels of lysine

Age in weeks	66% of lysine requirement in diet			100% of lysine requirement in diet		
	Liver fat % Wet tissue	% Fresh femur		Liver fat % Wet tissue	% Fresh femur	
		Water	Fat		Water	Fat
1	6.0	46.5	17.8	4.8	58.7	8.2
2	2.3	54.3	15.8	5.1	61.8	5.0
3	2.9	43.4	21.7	3.0	62.5	5.1
4	2.8	50.4	18.8	3.0	57.4	6.6
5	3.2	52.9	14.2	2.7	57.2	7.1
6	2.6	49.1	12.5	2.9	59.8	7.3
7	2.6	52.1	10.8	2.7	56.1	9.8

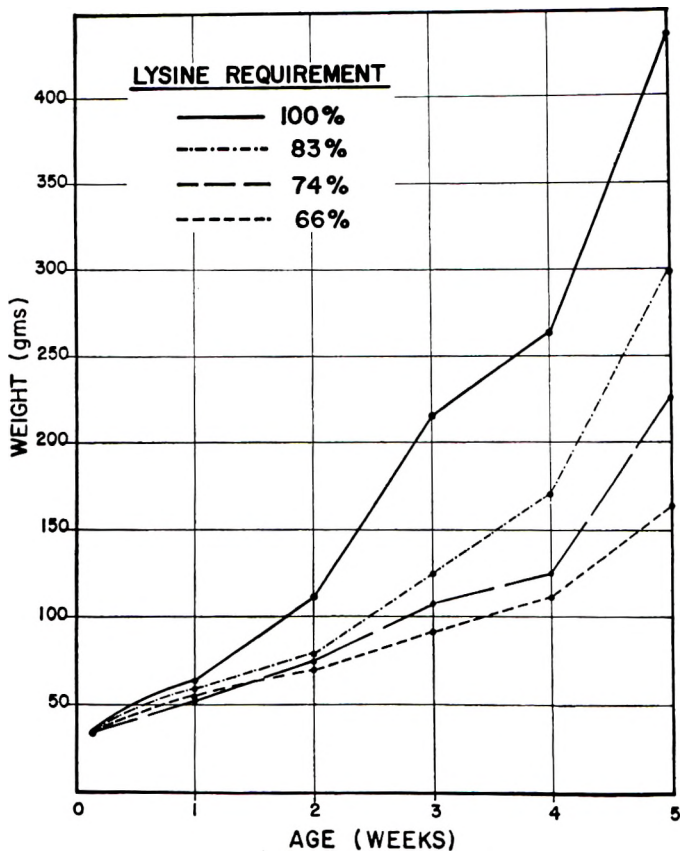

Fig. 2 Weight of chicks fed different levels of lysine (averages for two experiments, 8 chicks/week/diet).

chemical findings as summarized in figure 3. The curve on the left shows the total fat content of the bone as determined by chemical methods and the one on the right the percentage of estimated myeloid tissue in the bone marrow. The fat increase in the bones of the birds fed diets low in lysine was due to fat replacing myeloid tissue.

The 4th column of table 5 presents, in microns, the average thickness of the compact bone at the middle part of the diaphysis. These figures demonstrate that the bone increased in length and thickness in direct proportion to the lysine level of the ration and the body size of the animals. In plate 2 (figs. 8–11) is shown a cross section of the diaphysis of 4 chicks, one from each group during the last week of treatment. There was an increased thickness of the cortical bone as the lysine level of the ration increased. In the last column of table 5 data is given on the thickness of the epiphyseal cartilage plate which confirm the retarded endochondral ossification in lysine deficient animals, previously reported by Haggar et al. ('55).

DISCUSSION

Pérez ('55) demonstrated a growth retardation and retarded bone maturation in children from lower socioeconomic groups of Central America. These changes appear not only in children suffering from severe protein malnutrition (kwashiorkor), but also in apparently healthy children of the same socioeconomic background. The protein intake of these children is low, mainly of vegetable origin (Flores et al., '60) and presumably deficient in lysine and trypto-

TABLE 2

Chemical composition of femurs of New Hampshire chicks (experiment 3)

Age	Lysine in diet, % of requirement	Av. body weight	Femur weight	Femur weight	Femur length	Water	% of fresh femur		% of ash	
							Ash	Fat	Calcium	Phosphorus
		gm	*gm*	*% of body weight*	*cm*					
1 Day	—	34	0.1926	0.58	2.45	68.2	9.1	3.6	29.4	18.8
1 Week	66	50	0.2538	0.51	2.68	55.9	10.9	13.1	33.0	19.0
	74	48	0.2426	0.50	2.66	54.5	9.8	14.3	32.8	18.8
	83	54	0.2289	0.42	2.57	53.5	11.9	11.3	33.0	20.0
	100	57	0.2614	0.45	2.61	55.8	10.7	10.7	32.7	18.8
2 Weeks	66	63	0.3676	0.59	3.03	55.2	11.8	15.8	33.3	16.8
	74	70	0.3671	0.52	2.96	56.0	12.4	11.4	33.3	17.3
	83	78	0.4226	0.54	3.12	55.1	12.7	11.0	33.1	17.3
	100	112	0.5909	0.52	3.26	60.9	12.2	6.1	33.4	17.0
3 Weeks	66	89	0.4699	0.53	3.33	51.9	11.3	17.1	34.1	17.4
	74	94	0.5146	0.54	3.37	54.2	12.2	12.9	32.9	17.7
	83	114	0.6587	0.57	3.74	53.9	12.8	12.6	33.3	17.2
	100	179	1.1072	0.62	3.97	61.2	11.8	4.9	33.6	16.9
4 Weeks	66	104	0.6801	0.65	3.79	51.4	13.5	16.2	34.0	17.2
	74	122	0.7924	0.66	3.88	52.7	13.3	14.2	34.1	17.3
	83	168	1.0656	0.63	4.19	53.9	13.7	10.7	34.6	17.4
	100	275	1.8053	0.65	4.64	58.3	13.3	6.9	34.4	18.2
5 Weeks	66	134	0.8312	0.62	4.18	52.8	14.1	14.8	33.0	18.2
	74	152	0.9854	0.64	4.35	55.1	13.8	11.7	33.9	18.5
	83	217	1.3670	0.62	4.68	55.7	13.7	10.0	34.8	18.3
	100	398	2.7842	0.70	5.58	57.7	13.2	8.8	33.6	17.3

TABLE 3

Chemical composition of femurs of New Hampshire chicks (experiment 4)

Age in weeks	Lysine in diet, % of requirement	Av. body weight	Femur weight	Femur weight	% of fresh femur			
					Water	Ash	Fat	Protein
		gm	*gm*	*% of body weight*				
1	66	56	0.2732	0.48	60.3	9.2	7.2	18.5
	74	56	0.2696	0.48	60.2	11.3	6.6	17.9
	83	61	0.3055	0.49	59.8	11.4	7.1	18.3
	100	68	0.3356	0.50	60.0	10.7	6.7	18.0
2	66	74	0.4104	0.53	51.2	13.5	17.1	15.1
	74	80	0.4466	0.54	55.2	12.0	13.3	16.1
	83	105	0.5706	0.56	58.1	11.6	10.3	16.1
	100	113	0.5943	0.52	60.7	11.6	6.9	16.9
3	66	93	0.5906	0.64	54.7	11.7	14.4	14.4
	74	123	0.6957	0.56	56.7	14.1	8.3	15.4
	83	156	0.9128	0.58	58.0	12.6	9.0	15.3
	100	253	1.5874	0.62	61.2	12.4	4.8	16.7
4	66	119	0.7120	0.59	47.0	13.3	20.5	15.2
	74	129	0.7406	0.57	49.3	13.6	18.2	15.0
	83	173	1.3441	0.63	56.1	13.3	10.1	15.8
	100	252	1.7278	0.68	59.9	12.3	6.8	16.7
5	66	194	1.3149	0.67	48.5	14.2	18.2	15.3
	74	298	2.0003	0.67	51.0	14.0	15.6	15.8
	83	379	2.8427	0.75	54.5	13.0	11.6	16.6
	100	477	3.6443	0.74	54.7	12.3	12.3	15.7

TABLE 4

Hemoglobin and hematocrit values of chicks fed different levels of lysine (experiment 4)

Age in weeks	Lysine in diet, % of requirement			
	66	74	83	100
	Hemoglobin gm/100 ml			
3	7.5 (4)[1]	5.9 (3)	7.7 (2)	9.4 (2)
4	7.7 (3)	7.2 (3)	8.5 (3)	8.9 (4)
5	7.8 (4)	7.8 (4)	8.7 (2)	8.9 (3)
	Hematocrit, %			
3	27.2 (4)	23.7 (3)	29.5 (2)	33.0 (2)
4	28.0 (3)	26.0 (4)	32.0 (3)	31.7 (4)
5	28.0 (4)	29.0 (4)	29.5 (2)	30.7 (3)

[1] Figures in parentheses indicate number of chicks sampled.

phan. Another characteristic of kwashiorkor is an anemia which is normocytic or slightly macrocytic unless iron deficiency is also present; the bone marrow in these cases has been found to be hypoplastic. The anemia responds favorably to the administration of diets high in good quality protein (Scrimshaw et al., '56).

Although these signs may be due to the total protein deficiency, the possibility that lysine deficiency may play an important role in their development cannot be overlooked. It was considered important, therefore, to determine the effect of the deficiency of this amino acid on the bone growth and chemical composition, as well as on the histological changes observed in the bone marrow of experimental chicks. From the experimental data obtained here, the following conclusions can be drawn: deficiency of lysine in chicks resulted in depressed total body and bone growth and in decreased hematocrit and hemoglobin values. Although it might be suspected that these changes could be attributed to negative nitrogen balance and not to lysine

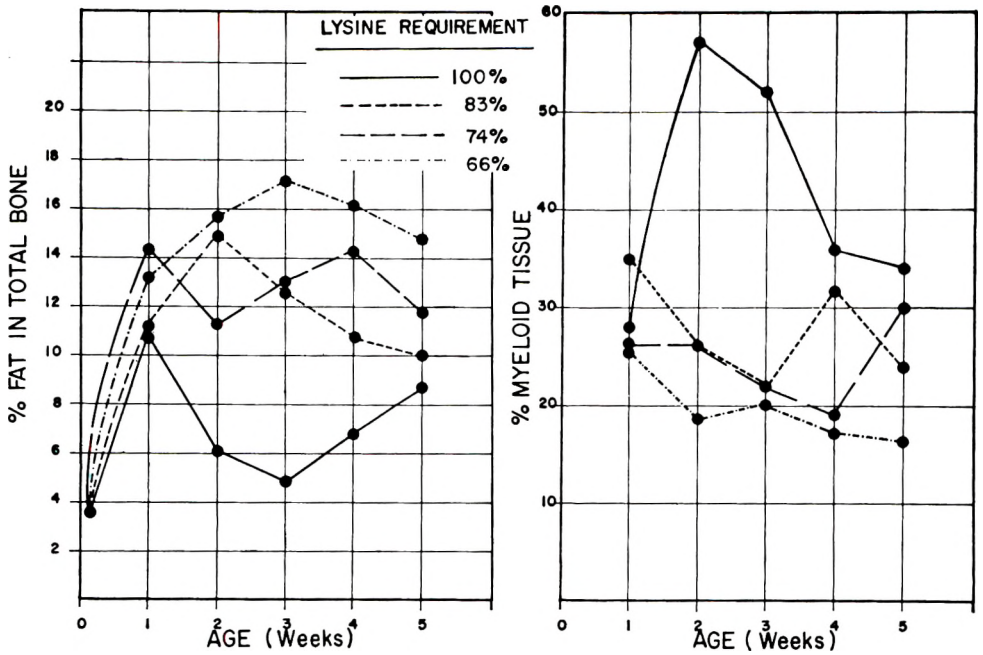

Fig. 3 Fat in total bone and myeloid tissue of chicks fed different levels of lysine (4 chicks/week/diet).

TABLE 5

Percentage of myeloid tissue and thickness of compact bone and epiphyseal plate of bones of New Hampshire chicks (experiment 3)

Age in weeks	Lysine in diet, % of requirement	% of myeloid tissue[1]	Thickness of compact bone	Thickness of epiphyseal plate
			mμ	mμ
1	66	26	182	1251
	74	26	159	731
	83	35	208	940
	100	28	224	1562
2	66	18	282	1124
	74	26	249	1085
	83	26	253	1229
	100	57	452	1857
3	66	20	272	1046
	74	22	292	927
	83	22	403	1518
	100	52	684	2388
4	66	17	286	773
	74	19	341	1124
	83	32	443	1750
	100	36	681	2292
5	66	16	325	1133
	74	30	340	1168
	83	24	450	1526
	100	34	663	1754

[1] The difference to 100% represents fatty tissue.

deficiency *per se*, the deficiency of lysine was not enough to cause negative nitrogen balance; instead, the animals in all of the experimental groups showed an increased body weight throughout the experimental periods (fig. 1).

The changes in the bone marrow appeared to be due to adipose tissue replacing shrinking amounts of myeloid tissue with a corresponding increase in total fat content of the bone and a decrease in its water and protein content. The resulting reduced activity of the bone marrow would eventually produce the low hemoglobin and hematocrit values reported in this and other studies (Gillespie et al., '45). Since the histological architecture of the bone was well preserved and its relative mineral composition did not change appreciably, it appears that lysine deficiency, *per se*, in an otherwise complete diet, did not interfere with either absorption or deposition of calcium salts.

In the animals fed 100% of the lysine requirement in experiment 3, the increase in percentage of myeloid tissue observed in the second and third weeks, and the decrease in older chicks could be due to yellow bone marrow replacing normal bone marrow. In the deficient chicks, these changes were not observed since the myeloid tissue is hypoplastic. The same argument applies to the epiphyseal plate which was thicker during the third and 4th weeks in the birds fed their complete lysine requirement.

It has been reported that the deficiency of lysine results in fatty livers in the rat (Singal et al., '53); however, these results were obtained with diets of very low protein content. In the present chick studies, despite the low levels of lysine fed, no fatty livers were observed due probably to the high protein content of the experimental rations used.

SUMMARY

The effect of lysine deficiency on the chemical and histological composition of the femurs of New Hampshire chicks, fed rations containing different levels of lysine, was studied. As the lysine level of the rations decreased there was a corresponding increase of fat in the femur and a decrease in its water content and in total body weight. Total protein content of the femur was reduced, but the percentage of ash and its calcium and phosphorus content did not change under the various treatments. Hemoglobin and hematocrit values were lower in those animals receiving low levels of lysine in the diet.

The histological studies showed that, as the lysine level of the rations decreased, there was a corresponding increase in the fatty tissue and a decrease in the myeloid tissue of the bone marrow. The thickness of the compact bone and that of the epiphyseal junction increased as the lysine level of the ration increased. The possible relationship of these findings to certain characteristics of severe protein malnutrition in children (kwashiorkor) was discussed.

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PLATE 1

EXPLANATION OF FIGURES

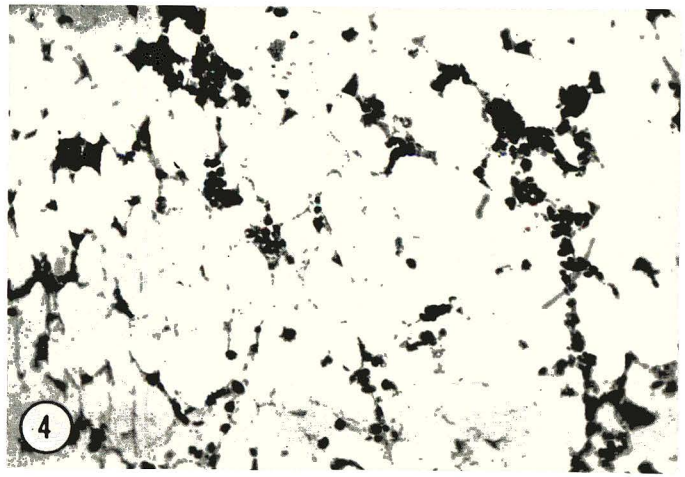
4 Fatty and myeloid tissues of the bone marrow of the femur of a chick fed 66% of its lysine requirement.

5 Fatty and myeloid tissues of the bone marrow of the femur of a chick fed 74% of its lysine requirement.

6 Fatty and myeloid tissues of the bone marrow of the femur of a chick fed 83% of its lysine requirement.

7 Fatty and myeloid tissues of the bone marrow of the femur of a chick fed 100% of its lysine requirement.

Bone marrow of the femur of a three-week-old chick fed, (4) 66, (5) 74, (6) 83, and (7) 100% of its lysine requirement. Observe the increase in myeloid tissue when the lysine level of the ration was raised. PTA Mallory stain. × 125.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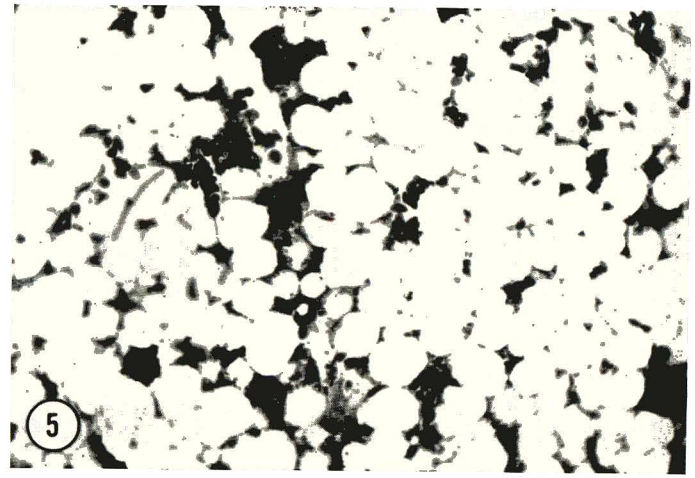
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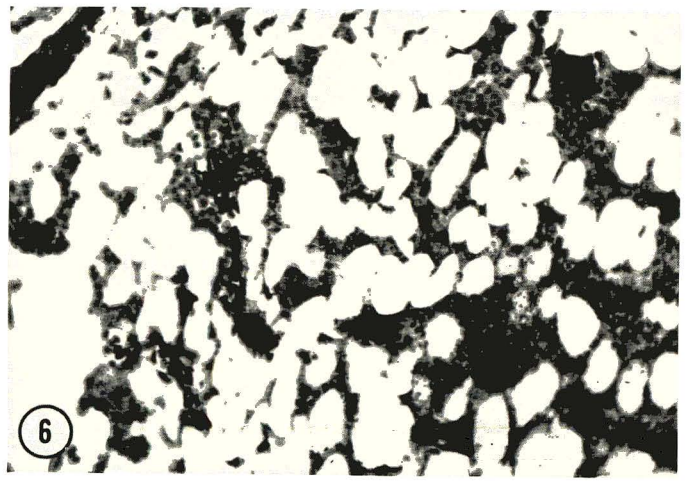
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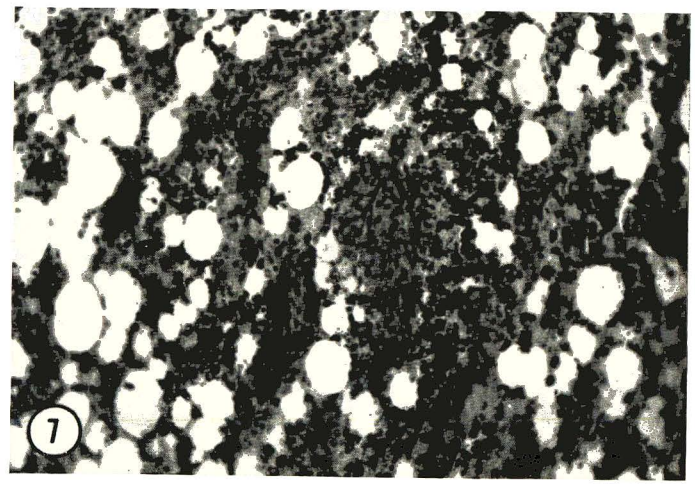
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PLATE 2

EXPLANATION OF FIGURES

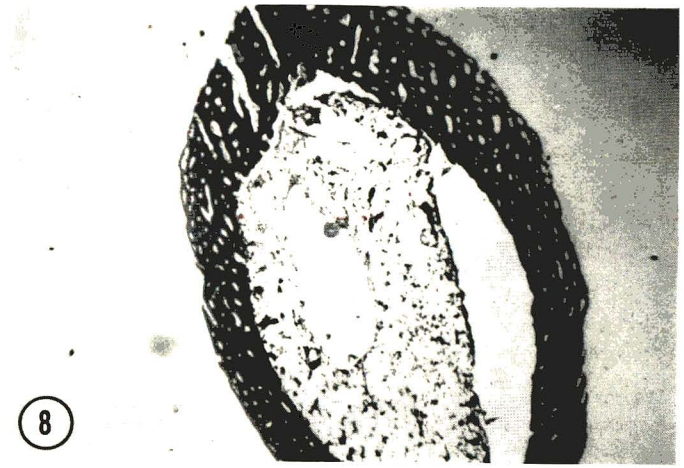
8 Cross section of the diaphysis of the femur of a chick fed 66% of its lysine requirement showing the compact bone and the bone marrow.

9 Cross section of the diaphysis of the femur of a chick fed 74% of its lysine requirement showing the compact bone and the bone marrow.

10 Cross section of the diaphysis of the femur of a chick fed 83% of its lysine requirement showing the compact bone and the bone marrow.

11 Cross section of the diaphysis of the femur of a chick fed 100% of its lysine requirement showing the compact bone and the bone marrow.

Cross section of the diaphysis of the femur of a 5-week-old chick fed, (8) 66, (9) 74, (10) 83 and (11) 100% of its lysine requirement. Note the increased thickness of the cortical bone when the lysine level of the ration was raised. PTA. Mallory stain. × 40.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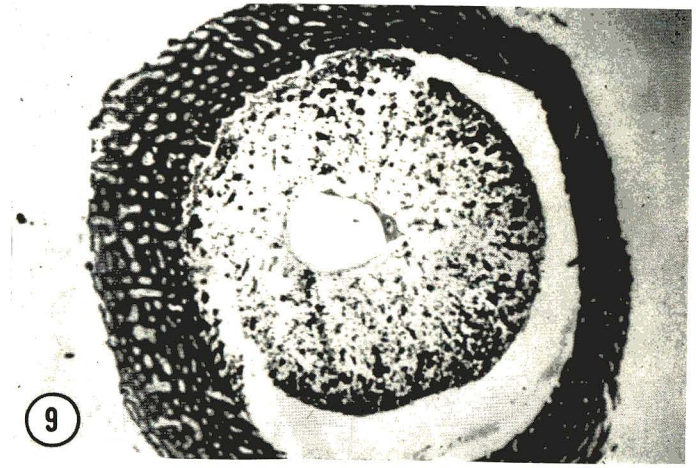
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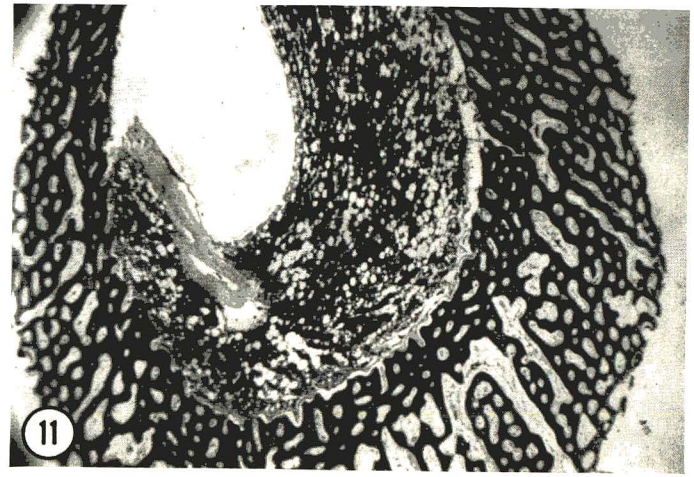
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Importance of Dispensable Amino Acids for Normal Growth of Chicks[1]

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The problem of devising diets containing free amino acids in place of protein continues to engage the attention of workers in the field of amino acid nutrition but as yet few of the diets described for chicks have proved capable of supporting a rate of growth approaching that of chicks fed a diet containing an adequate amount of well-balanced protein.

The mixture of 12 amino acids which Hegsted ('44) fed to chicks supported a rate of gain of only about 3.5 gm per day, as contrasted with 8 to 10 gm per day for chicks fed a control diet containing casein. Almquist and Grau ('44), while determining amino acid requirements, observed that the growth rate of chicks 10 to 14 days old decreased to about 4 gm per day when a purified diet containing 20 amino acids as the nitrogen source was substituted for a commercial diet. Grau and Peterson ('46) reported growth rates of only 2 to 4 gm per day for chicks fed their amino acid diet. Luckey et al. ('47) found that none of the different amino acid diets they fed to chicks supported a growth rate comparable to that of a control group fed a purified diet containing casein.

Amino acids diets used by Benton et al. ('55a, b, '57), which contained the indispensable amino acids in quantities equal to 1.5 times the accepted requirements (Almquist, '52) of the chick and some dispensable amino acids as well, supported a growth rate of only 4 gm per day; however, this was increased to about 8 gm per day when the diet was supplemented with 5 to 10% of gelatin. Fisher and Johnson ('57) found that the growth rates of chicks fed either an amino acid diet or a corn-soybean diet were similar, of the order of 6 gm per day. The growth rate of the control group receiving the natural diet, however, was lower than would normally be expected. Fox et al. ('58) reported that the growth rate of chicks fed an amino acid diet described by Fisher and Johnson ('56) was equivalent, at least for two weeks, to that of a control group fed a casein-gelatin diet. In this work, also, the growth of the group fed the diet containing protein was only about 6 gm per day for the first two weeks of the experiment. Klain et al., ('60), in a recent paper on the amino acid requirements of the chick, reported growth rates of about 9 gm per day for chicks receiving no intact protein. The chicks they used, however, were fed a diet containing protein for the first 7 days after hatching.

The study described below was originally undertaken to determine why gelatin stimulated the growth of chicks fed an amino acid diet (Benton et al., '55a, b). Attempts to isolate an active material from gelatin were unsuccessful but some fractions were obtained which enhanced growth slightly.[2] These fractions contained large amounts of amino acids and although neither a larger amount of the basal amino acid mixture nor additions of individual amino acids stimulated growth, 5% of a mixture of glycine, proline and glutamic acid did. At about the same time Klain et al. ('59) reported that large supplements of glutamic acid stimulated the growth of chicks fed an amino acid diet having a high ratio of indispensable to

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[2] Stucki, W. P. 1959 Studies on factors in gelatin affecting the growth of chicks on amino acid diets. M. S. thesis, University of Wisconsin, Madison, Wisconsin.

dispensable amino acids. An effort was made therefore, to estimate the most satisfactory ratio of indispensable to dispensable amino acids in amino acid diets for chicks.

EXPERIMENTAL

Animals

Straight-run Vantress chicks obtained from a commercial hatchery were used in all experiments. They were maintained in electrically heated brooder cages with raised wire-mesh floors. Fresh running water was supplied and feed was given ad libitum. The day-old chicks were separated into groups on the basis of body weight. They were starved for 48 hours then fed the basal amino acid diet for three days to deplete them of possible growth factors from the yolk sac. They were then weighed again and those that had gained either much more or much less weight than the average for their respective groups were discarded. Experimental groups of 7 chicks each were selected so that the weight distribution for each group resembled that for the whole population and so that the average weights of all groups were similar. Average weights ranged from 45 to 50 gm in the different experiments, about 10 gm above the initial average weight. The birds were weighed at the start of the experiment, at three and 7 days, and at the end of the experiment at 10 days.

Diets

The diets used in all experiments were devoid of protein but contained, in almost all cases, amounts of indispensable amino acids sufficient to meet the accepted requirements of the chick (Almquist, '52). The composition of the basal diet was as follows, in per cent: amino acid mixture, 23.36; dextrin (moist cornstarch heated for two hours at 115° in an autoclave, then dried and ground), 64.85; salts (Briggs et al., '43), 6.00; vitamin[3] mixture (Benton et al., '57), 0.62; choline chloride, 0.17; and soybean oil, 5.00. α-Tocopheryl acetate was supplied in the soybean oil, and vitamins A and D_3 were supplied by weekly supplementation with fortified halibut liver oil.

The amino acid mixture, as used in the basal diet, supplied the following amounts of amino acids, as percentage of the diet: L-arginine monohydrochloride, 2.30; glycine, 1.50; L-histidine monohydrochloride monohydrate, 0.55; DL-isoleucine (50% L-isoleucine), 1.80; L-leucine, 2.10; L-lysine monohydrochloride, 1.65; DL-methionine, 1.35; DL-phenylalanine, 1.40; DL-threonine, 1.80; DL-tryptophan, 0.76; DL-valine, 2.40; DL-alanine, 0.50; DL-aspartic acid, 0.50; L-cystine, 0.20 (an additional 0.30% is supplied by the vitamin mixture); L-glutamic acid, 2.50; L-proline, 0.50; DL-serine, 0.50; and L-tyrosine, 1.05. The amount of each L-indispensable amino acid equaled 1.5 times the accepted requirement, whereas each dispensable amino acid was supplied at a level equal to or greater than 0.5% of the diet. All additions to the diets were made at the expense of either the dextrin, when the total nitrogen content of a particular diet was to be raised, or the basal amino acid mixture, when the amino acid composition was to be varied while maintaining the nitrogen content of the diet constant within a particular series of experimental groups.

The amino acid compositions of the diets used in this work are presented in tables 1 and 2. The I/D ratio is defined as the ratio of nitrogen contributed by the indispensable amino acids to the nitrogen contributed by the dispensable amino acids, and was calculated on the following basis. (a) All nitrogen contributed by both isomers of the racemic indispensable amino acids, as well as the nitrogen contributed by the guanidino nitrogen of arginine and the epsilon-amino nitrogen of lysine was considered "indispensable nitrogen." (b) All nitrogen contributed by the dispensable amino acids, including both isomers of the racemic amino acids, was considered "dispensable nitrogen." (c) All nitrogen originating from glycine was considered "indispensable nitrogen." This method of computing the I/D ratio is arbitrary but was selected because of the limited quantitative information about the utilization of the D-isomers of the amino acids.

[3] Some of the crystalline vitamins were kindly supplied by Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

TABLE 1

Growth rates of chicks fed an amino acid diet supplemented with gelatin or amino acids

Diet composition	Nitrogen content	Growth rate[1]
	% of diet	*gm/day*
1 Basal diet (23.4% basal amino acid mixture)	3.05	5.3 ± 0.5
2 As in (1) + 1% glycine + 1% proline + 3% glutamic acid	3.64	8.2 ± 0.8
3 As in (1) + additional basal amino acid mixture to make (3) isonitrogenous with (2)	3.64	5.4 ± 0.3
4 As in (1) + 5% gelatin	—	7.6 ± 0.4
5 18.8% Basal amino acid mixture + 1% glycine + 1% proline + 3% glutamic acid	3.05	7.0 ± 0.5

[1] The mean ± the standard error of the mean.

TABLE 2

Amino acid composition of the diets and final body weights of chicks fed the diets

Diet	I/D ratio	Basal amino acid mixture	Supplementary amino acid mixture[1]	Nitrogen	Final body weight
		%	%	*% of diet*	*gm*
			Experiment 1		
1	3.2	17.85	1.05	2.45	82 ± 4[2]
2	2.8	17.03	1.95	2.45	82 ± 3
3	2.4	16.04	3.05	2.45	81 ± 8
4	2.0	14.79	4.43	2.45	81 ± 5
5	3.2	22.18	1.30	3.05	84 ± 5
6	2.8	21.19	2.40	3.05	86 ± 7
7	2.4	19.97	3.75	3.05	102 ± 5
8	2.0	18.38	5.50	3.05	86 ± 5
9	3.2	26.51	1.55	3.64	91 ± 5
10	2.8	25.34	2.85	3.64	108 ± 3
11	2.4	23.85	4.50	3.64	104 ± 7
12	2.0	21.99	6.55	3.64	121 ± 12
13	3.2	30.85	1.80	4.24	98 ± 6
14	2.8	29.45	3.35	4.24	107 ± 10
15	2.4	27.73	5.25	4.24	115 ± 6
16	2.0	25.58	7.62	4.24	127 ± 5
			Experiment 2		
17	3.8	23.36	—	3.05	90 ± 5
18	2.3	23.36	5.00	3.64	118 ± 12
19	1.6	19.26	9.50	3.64	110 ± 8
20	1.2	16.19	12.88	3.64	90 ± 4
21	3.8	37.03	—	4.83	90 ± 8
22	2.8	33.50	3.88	4.83	112 ± 5
23	2.0	29.13	8.68	4.83	120 ± 7
24	1.6	25.93	12.20	4.83	126 ± 9
25	1.2	21.56	17.00	4.83	108 ± 4

[1] Mixture of glycine:proline:glutamic acid (1:1:3).
[2] The mean ± the standard error of the mean.

RESULTS

The results of a typical experiment in which the effect of adding 5% of a glycine-proline-glutamic acid (1:1:3) mixture to the basal diet was studied are shown in table 1. Whenever the mixture has been tested it has enhanced growth as much or more than an equivalent supplement of gelatin.

As the mixture was composed primarily of dispensable amino acids, subsequent experiments were designed to test the effect of varying the ratio of indispensable to dispensable amino acids in a series of diets

having different nitrogen contents. The experimental design and the results are given in table 2. From the final body weights in the last column, it may be seen that lowering the ration of indispensable to dispensable amino acid nitrogen (I/D) from 3.2 to 2.0, particularly in diets in which the level of nitrogen was so low that the amino acid requirements were barely being met, had little, if any, effect on the growth rate or final body weight of chicks. As the nitrogen level was increased, the effect of lowering the ratio was more marked; that is, a relatively small drop in I/D ratio resulted in a relatively large increase in final body weight.

If the final average body weights as given in the table for the groups in experiment 1 and the adjusted (see later) final average body weights of the groups in experiment 2 are plotted in the third dimension with nitrogen content and I/D ratio of the diet as the first and second dimensions, a figure approximately represented by figure 1 is generated.

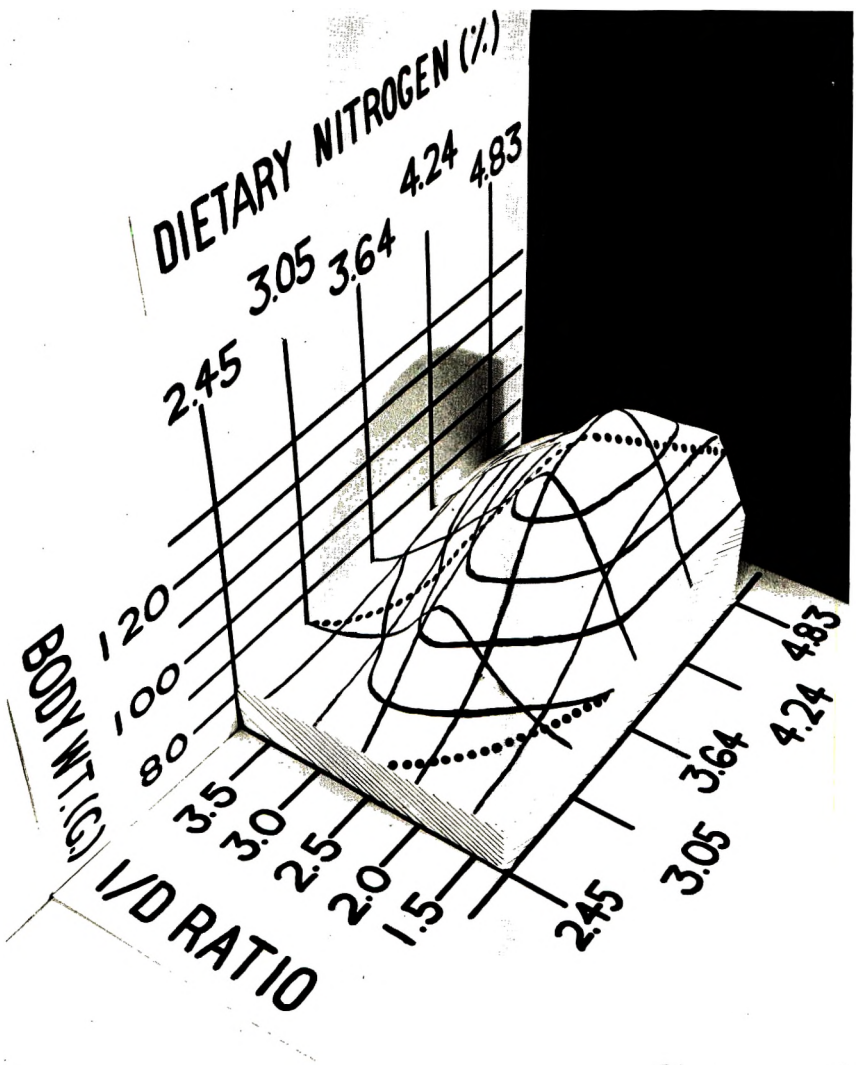

Fig. 1 Three-dimensional plot of growth of chicks versus nitrogen content of the diet and ratio of indispensable to dispensable amino acids.

Experiments 1 and 2 were not run at the same time but groups 11 and 18, were fed diets containing the same amount of nitrogen (3.64%) and having similar I/D ratios. The average final body weight of group 11 was 0.88 times that of group 18, and therefore the final body weights for all groups in experiment 2 were multiplied by 0.88 in order to put both experiments on a common basis. Although the validity of this procedure may be questioned it should be noted that the body weight values for groups 17 and 21 (3.8 ratio) multiplied by 0.88 describe a line in the body weight-nitrogen level plane which meets that described by the values for groups 1 through 4 in the body weight-I/D ratio plane where the nitrogen level is 2.45%.

The grid markings on the surface of the figure correspond to the coordinates shown on the background and the base. An approximation to the appearance of a cross-section of the figure through the line of maximum final body weight is provided by the shadow projected on the background. The baseline for body weight has been set at 70 gm, in order to emphasize the differences among the various groups. At points on the shorter dotted line passing across the nearest corner of the surface the requirements of the chick for the indispensable amino acids (not including glycine, since it is supplied in the supplementary amino acid mixture) were just met. At points on the longer dotted line 1.5 times the requirements for the indispensable amino acids were supplied. Therefore, at all points between the near corner and the shorter dotted line the amounts of indispensable amino acids in the diet were below the accepted requirements; between the two dotted lines the diets contained 1.0 to 1.5 times the requirements for the indispensable amino acids; and between the longer dotted line and the far corner the diets contained more than 1.5 times the requirements.

This figure emphasizes the fact that increasing the nitrogen content of the diet had no effect on growth when the I/D ratio was 3.8; that is, when the proportional composition of the amino acid mixture remained the same as that in the basal diet. It also shows that changing the I/D ratio from 3.2 to 2.0 had no effect on growth when the nitrogen level of the diet was kept at 2.45%; at higher levels, however, the effect of a change in ratio was marked.

Close examination of figure 1 will show that the I/D ratio at which maximum growth occurred at any given nitrogen level changed slightly as the amount of dietary nitrogen was increased. At the lowest nitrogen level studied no maximum occurred, possibly because the requirements for the indispensable amino acids were not met by diets containing only 2.45% of nitrogen except when the I/D ratio was high. The values for the groups receiving the highest dietary level of nitrogen were determined in a separate experiment so the apparent decline in growth when the nitrogen content of the diet was increased from the second highest to the highest level must be accepted with reservation.

Maxima in final body weight did occur in each of the series with higher nitrogen intakes, indicating that the ratio of indispensable to dispensable nitrogen cannot be varied widely without influencing growth. From these results it appears that the optimum I/D ratio changes from about 2.4 in the diet containing 3.05% of nitrogen to 1.5 in that containing 4.83% of nitrogen.

DISCUSSION

It is apparent from the results presented that mere satisfaction of the requirements for the indispensable amino acids is not the only criterion which must be met in order to obtain maximum growth of chicks fed protein-free diets in which the nitrogen is supplied by amino acids. This was demonstrated earlier by Luckey et al. ('47), who stated that "diets containing some of the non-essential amino acids are superior to diets containing the 11 amino acids now recognized as essential for the chick." Similar conclusions have also been drawn concerning the diet of the rat by Lardy and Feldott ('50), Frost and Sandy ('51), Rechcigl et al. ('57), and others. Frost and Sandy ('51) concluded that rats grew most rapidly when fed diets in which about 20% of the nitrogen was supplied by sources other than indispensable amino acids.

The optimum I/D ratio for the chick, as determined in this study, was approximately 2.0 for diets containing from 3.64 to 4.24% of nitrogen; namely, about 33% of the dietary nitrogen had to be supplied as dispensable amino acids in order to obtain maximum growth. The method used for calculation of the I/D ratio, however, was arbitrary and at least three alternatives exist for the disposition of the D-isomers of the racemic indispensable amino acids. (1) They may be included with the respective L-isomers. (2) They may be considered to contribute only dispensable or nonspecific nitrogen. (3) They may be considered to provide both dispensable and indispensable nitrogen, depending on the particular amino acids involved. Although the last alternative is undoubtedly correct, a true I/D ratio cannot be calculated because accurate information on the disposition of the D-forms is not available. The extremes represented by the first two alternatives should give a reasonable approximation of the upper and lower limits for the dispensable nitrogen requirement of the chick.

Calculation of the I/D ratio for diet number 16, the diet that supported the greatest growth rate, using alternative (2) but including D-methionine with the indispensable amino acids, yields a value of 1.2 as compared with the value of 2.0 obtained by alternative (1). Thus the extreme values for the dispensable nitrogen content of this diet are 33 and 45%. The high value agrees unexpectedly well with the estimate made by Rosenberg ('59).

One factor is still not taken into account in these calculations. Excess glycine was considered as a source of indispensable nitrogen throughout this study but at some definite glycine content the excess can probably no longer be classified as indispensable and should be included with the dispensable amino acids. Thus the range given for the optimal amount of dispensable nitrogen is probably a little large.

As the nitrogen content of the diet, or, in essence, the "protein" content of the diet was increased (fig. 1), the proportion of the indispensable amino acids required for maximum growth decreased. The lysine and total sulfur amino acid requirements of the chick, expressed as a percentage of the dietary protein, decrease as the protein level of the diet is raised (Almquist, '52), and it was suggested that the same relation would also apply to the other indispensable amino acids. The present work provides further evidence of the relationship suggested above. In addition, it demonstrates that too high a proportion of indispensable nitrogen in the diet is inhibitory regardless of the nitrogen level of the diet and that a low I/D ratio may also depress growth even when adequate amounts of indispensable amino acids are being supplied.

The results of these experiments also suggest a possible explanation for the growth-promoting effect of gelatin reported by Benton et al. ('57). The basal diet used both in this work and in the work of Benton et al. was characterized by having a relatively large content of indispensable amino acids as compared with the content of the dispensable amino acids. Benton et al. ('55a) found that supplementation of the basal diet with gelatin or zein proved superior to supplementation with casein or fibrin. If glycine is considered to be a dispensable amino acid, or, as a compromise, considering the lack of knowledge about its function, is entirely neglected in the ratio calculation, then gelatin and zein have calculated I/D ratios of about 0.7. Casein and fibrin, on the other hand, have ratios of about 1.2 and 1.5, respectively. Additions of gelatin and zein would reduce the I/D ratio of the diet considerably more than equal additions of casein and fibrin and, thus, would be expected to stimulate growth more than casein or fibrin. To answer this question unequivocally, diets containing L-amino acids only, would be necessary in order to eliminate the uncertainty involved in the calculation of I/D ratios.

Finally, a comment should be made regarding the adequacy of the best of these amino acid diets for the growing chick. The average growth rate of 8.0 gm per day is certainly below the rate of about 14 gm per day that can be obtained with a well-balanced control diet over a comparable 10-day period. Young growing ani-

mals, however, require a period of adaptation to amino acid diets so the growth rates of 10 to 11 gm per day, obtained toward the end of the first week, for chicks receiving no protein from time of hatching are considered quite satisfactory. The diet has not been tested in experiments longer than two weeks.

SUMMARY

A study was undertaken to estimate the optimal dietary ratio of indispensable to dispensable amino acids (the I/D ratio) for chicks fed amino acid diets. Graded levels of a glycine-proline-glutamic acid mixture were added, at the expense of the basal amino acid mixture, to diets having different total nitrogen contents. Growth results were plotted versus nitrogen level and I/D ratio to produce a three-dimensional figure. Examination of the figure indicated that, under the conditions of these experiments, growth was greatest over a fairly wide range of dietary nitrogen levels when about 33% of the dietary nitrogen was supplied from dispensable amino acids. The results also suggest that gelatin stimulates the growth of chicks fed certain amino acid diets because of its high contribution of dispensable amino acids.

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Metabolism of Norleucine by the Intact Cow[1]

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The metabolic fate of norleucine in cows is of special interest since norleucine is apparently not a normal component of protein (Consden et al., '45; Black and Kleiber, '55) but is incorporated into casein when injected intravenously into cows (Black and Kleiber, '55). Norleucine is also incorporated into protein by *Escherichia coli* (Cowie et al., '59).

To learn more about the metabolic behavior of norleucine, cows were given increasing amounts of DL-norleucine intravenously for 90 days (referred to hereafter as the adaptive period).[3] Ten days before the adaptive period, and again immediately after, the cows were injected with DL-norleucine-2-C^{14}. The effect of this adaptive period on the oxidation of norleucine (indicated by the C^{14} level in respired CO_2) and on the utilization of norleucine carbon for biosynthesis (indicated by the C^{14} level in milk components) was reported earlier (Kaneko et al., '60). The change of specific activity among various components indicated that the adaptive period was followed by greater utilization of norleucine carbon for synthesis of milk components, greater oxidation of norleucine carbon to CO_2, and reduced loss of norleucine carbon via urinary acetone.

The present paper reports on the utilization of carbon from norleucine for the synthesis of casein amino acids, and how this is affected by the adaptive period. In addition, evidence is presented on the pathway for metabolism of norleucine in the intact cow.

EXPERIMENTAL

Four lactating cows were injected intravenously with a single dose of DL-norleucine-2-C^{14} or DL-norleucine-3-C^{14}. Data on the animals injected with norleucine-2-C^{14} (trials 3, 4, 5 and 6) were published elsewhere (Kaneko et al., '60); data on the animals injected with norleucine-3-C^{14} (trials 1 and 2) are in a footnote to table 1. Description has been given of the procedures for injecting the isotope (Ralston et al., '49), collecting and fractionating the milk into its components (Kleiber et al., '52), hydrolyzing casein (Black et al., '55), isolating amino acids from casein hydrolysate with ion-exchange columns (Black et al., '54), and assaying samples (Black et al., '55). Serine was degraded by the method of Sakami ('50), glutamic acid by the method of Mosbach et al. ('51) as modified by Koeppe and Hill ('55), and alanine by a method previously described (Black et al., '57).

The norleucine-2-C^{14} was synthesized in our laboratory by a method described elsewhere (Kaneko et al., '58). The norleucine-3-C^{14} was synthesized at the Radiation Laboratory, University of California.[4]

RESULTS AND DISCUSSION

In table 1 are shown the specific activities of amino acids recovered from casein three hours after cows were injected with norleucine-3-C^{14} or norleucine-2-C^{14}. In each trial the specific activity was greatest in glutamic acid and decreased in this se-

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[2] Present address: Western Regional Research Laboratory, Albany, California.
[3] Cows were given 1 gm of DL-norleucine daily by intravenous drip of an isotonic solution into the jugular vein. The daily dose was increased slowly, finally reaching a level of 20 gm per day by the 90th day of the adaptive period. The DL-norleucine was purchased from Nutritional Biochemicals Corporation, Cleveland.
[4] By R. Ostwald, P. T. Adams and B. Tolbert of the Bio-organic Group.

TABLE 1

Specific activities[1] of amino acids from casein three hours after injection of norleucine-2-C^{14} or norleucine-3-C^{14}

Trial no.	Compound injected	Amino acid						
		Glutamic acid	Aspartic acid	Serine	Alanine	Glycine	Proline	Arginine
1	Norleucine-3-C^{14}	8.02[2]	4.88	7.86	2.32	2.01	0.68	0.18
2	Norlecuine-3-C^{14}	6.37	3.86	4.64	1.94	1.63	0.5	0.07
3	Norleucine-2-C^{14}	0.97	0.42	0.38	0.18	0.21	—	—
5	Norleucine-2-C^{14}	1.53	0.68	0.47	0.17	0.37	—	—
4	Norleucine-2-C^{14}	1.92	0.91	0.46	0.45	0.48	—	—
6	Norleucine-2-C^{14}	2.32	1.04	0.81	0.42	0.53	—	—

[1] Specific activities are expressed as (microcuries of C^{14} per gram atom carbon) per unit injected dose (microcuries of C^{14} injected per kilogram of body weight). The cow used in trial 1, cow no. 941, weighed 470 kg and was injected with 3.62 mc of norleucine-3-C^{14}. The cow used in trial 2, cow no. 962, weighed 480 kg and was injected with 3.34 mc of norleucine-3-C^{14}. Data on cows injected with norleucine-2-C^{14}, trials 3 to 6, were published elsewhere (Kaneko et al., '60). One cow was used for trials 3 and 5 and another for trials 4 and 6. Trials 3 and 4 were conducted before the period when the cows received increasing amounts of unlabeled norleucine and trials 5 and 6 immediately after this interval was terminated.

[2] A specific activity of 8.02 gave 2200 net cpm with "infinite-thickness" $BaCO_3$ in our counting system.

quence: glutamic acid, aspartic acid or serine, alanine or glycine, proline and arginine.

If one compares the specific activities of the amino acids synthesized from norleucine-2-C^{14} before (trials 3 and 4) and after (trials 5 and 6) the adaptive period in which the cow received unlabeled norleucine, it is apparent that these specific activities increased by 15 to 50% for all amino acids except alanine. Based on the increased specific activity, the adaptive period appears to result in 15 to 50% greater utilization of norleucine carbon by the cow for synthesis of glycine, serine, glutamic and aspartic acids.

This change in utilization may have been even greater than the estimated value, because of the pool of unlabeled norleucine that accumulated in the cow's tissues during the adaptive period. The presence of this pool was indicated by the fact that the norleucine which was recovered from casein in trials 3 and 4 (prior to the adaptive period) had the same specific activity as the norleucine-2-C^{14} injected into the cow (see table 4, Kaneko et al., '60). After the adaptive period, however, the norleucine recovered from casein had specific activities that were only about 10% as great as the norleucine-2-C^{14} injected. If the norleucine-2-C^{14} that was metabolized during trials 5 and 6 underwent the same dilution by the pool of unlabeled amino acid as the norleucine that was incorporated into casein, one can estimate that the utilization of norleucine carbon was increased some 10 times during the adaptive period. It is possible that the dilution encountered by the norleucine that was incorporated into casein may not have been the same as that encountered by the norleucine that was metabolized and converted to other amino acids. Casein is formed in the udder of the cow, and the norleucine incorporated into casein may have been diluted by a pool largely in this organ. Since the other amino acids that contained C^{14} (table 1) can be formed in liver as well as udder, the norleucine carbon from which they were derived may not have undergone a tenfold dilution.

These considerations may change the estimated magnitude of the metabolic alteration resulting from the adaptive period, but not the fact that this period was followed by greater utilization of norleucine carbon for amino acid synthesis.

Studies with the intact rat (Hassan and Greenberg, '52) and with perfused rat liver (Kaneko et al., '61) have shown that norleucine is readily metabolized and that carbon from norleucine appears in products synthesized by that rat.

Incubation of rat liver homogenate with norleucine-3-C^{14} (Kinnory et al., '55) re-

sulted in the production of C^{14}-α-ketocaproate, C^{14}-valerate, C^{14}-β-hydroxyvalerate, C^{14}-acetate, and unlabeled propionate as products of the reaction in the medium. They concluded that the metabolic pathway for norleucine involved deamination, decarboxylation, and beta-oxidation of the valerate produced. If the same pathway is responsible for norleucine metabolism in the cow, one can predict that norleucine-2-C^{14} and norleucine-3-C^{14} would give rise, respectively, to acetyl CoA labeled in the C-1 and C-2 position of the acetyl moiety. This sequence is shown on figure 1.

To test whether this proposed sequence is responsible for norleucine metabolism in the intact cow, the labeling pattern in amino acids after injecting norleucine-C^{14} can be compared with the patterns obtained after injecting other compounds metabolized through a common intermediate. Butyrate offers such a possibility since it is known to undergo beta oxidation to form acetyl CoA (Weinhouse, '54). Thus butyrate-1-C^{14} or -3-C^{14} would yield acetyl CoA-1-C^{14} and butyrate-2-C^{14} would yield acetyl CoA-2-C^{14}, as indicated in figure 1.

In table 2 is shown the intramolecular C^{14} distribution in serine and alanine after injection of cows with norleucine or butyrate. Injection of norleucine-2-C^{14} or butyrate-1-C^{14} resulted in the formation of serine and alanine with C^{14} located principally in the C-1 position. This is the expected location when acetyl CoA-1-C^{14} is metabolized via the tricarboxylic acid cycle and the amino acids are synthesized from glycolytic intermediates according to the scheme shown in figure 1.

Norleucine-3-C^{14} and butyrate-2-C^{14} gave similar results in the labeling of serine (table 2). In both cases the C^{14} was located principally in the C-2 and C-3 positions,

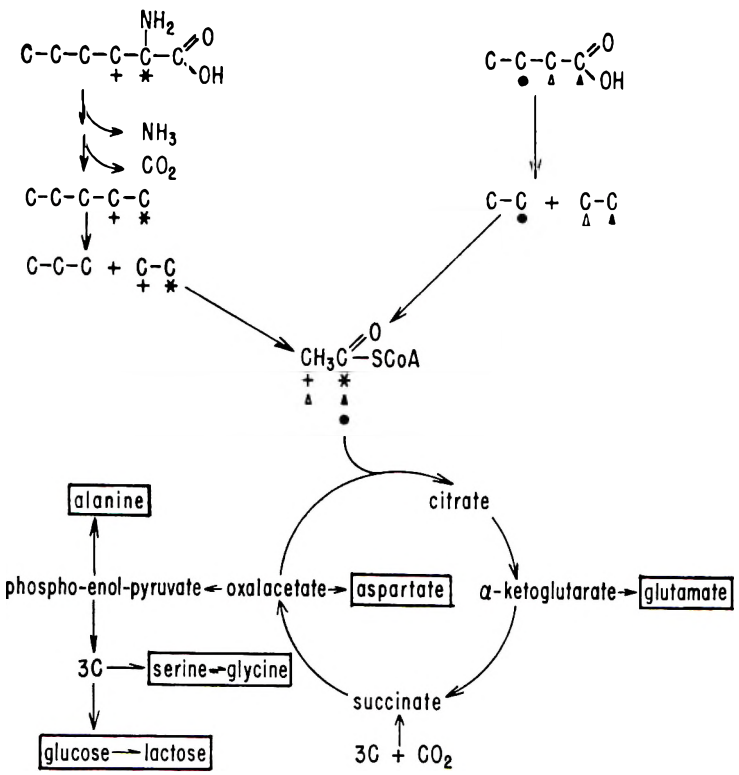

Fig. 1 Scheme outlining the relationship between acetyl CoA arising from norleucine or from butyrate. The origin of amino acids is indicated in respect to intermediates of the tricarboxylic acid cycle and glycolysis. Symbols denote the origin of individual carbon atoms from butyrate-C^{14} or norleucine-C^{14}.

TABLE 2

C^{14} distribution in serine and alanine after injection of norleucine-2-C^{14} and norleucine-3-C^{14}

Trial designation	Compound injected	Amino acid	Specific activity	% C^{14} in each carbon[1]		
				C-1	C-2	C-3
Norleucine 4	Norleucine-2-C^{14}	Serine	0.5	87	5.3	2.7
Norleucine 5	Norleucine-2-C^{14}	Serine	0.5	80	4	3.3
Butyrate 1	Butyrate-1-C^{14}	Serine	1.2	97.2	5	2
Norleucine 5	Norleucine-2-C^{14}	Alanine	0.2	67	12	17
Butyrate 1	Butyrate-1-C^{14}	Alanine	1.1	85		9.1[2]
Norleucine 1	Norleucine-3-C^{14}	Serine	7.9	11.0	37.2	40
Norleucine 2	Norleucine-3-C^{14}	Serine	4.6	13.0	39.2	45
Butyrate 3	Butyrate-2-C^{14}	Serine	3.4	15.6	39.4	43

[1] C^{14} in each carbon expressed as percentage of total in molecule determined by combustion of amino acid.

[2] C-2 and C-3 determined together.

TABLE 3

C^{14} distribution in glutamic acid after injection of norleucine-2-C^{14} and norleucine-3-C^{14}

Trial designation	Compound injected	% C^{14} in each carbon[1]					Specific activity of amino acid
		C-1	C-2	C-3	C-4	C-5	
Norleucine 4	Norleucine-2-C^{14}	23.2				71.6	1.9
Norleucine 6	Norleucine-2-C^{14}	25.2		1.7[3]		75.6	2.3
Butyrate 1[2]	Butyrate-1-C^{14}	25.4		0.7[3]		74.6	8.9
Butyrate 6[2]	Butyrate-3-C^{14}	26.0	0.3	0.2	0.1	75.4	6.0
Norleucine 2	Norleucine-3-C^{14}	9.4	20.6[4]	20.6[4]	46.0	2.8	6.4
Butyrate 3[2]	Butyrate-2-C^{14}	9.3	20.2	18.8	41.6	1.2	10.1

[1] C^{14} in each carbon atom in percentage of total C^{14} determined by complete combustion of glutamic acid.

[2] These data have been reported elsewhere (Black et al., '61) but are repeated here for convenience in comparing results after norleucine-C^{14} and butyrate-C^{14}.

[3] C^{14} measured in C-2 plus C-3 plus C-4.

[4] C-2 and C-3 determined together. C^{14} assumed to be equally distributed between the two carbons.

with about equal amounts of C^{14} in each carbon, and one-third to one-half as much C^{14} in C-1.

Further evidence of the close agreement between results with norleucine and butyrate is given by the labeling pattern in glutamic acid (table 3). The C^{14} from norleucine-2-C^{14} and from butyrate-1-C^{14} or butyrate-3-C^{14} is located principally in the C-1 and C-5 positions of glutamic acid, with about three times as much in C-5 as in C-1. Similarly, the intramolecular C^{14} in glutamate after norleucine-3-C^{14} corresponds with that after butyrate-2-C^{14}.

The relative specific activities among the amino acids provide another indication of the pathway for norleucine metabolism. According to the scheme of the tricarboxylic acid cycle, the carboxyl carbon of the acetyl moiety of acetyl CoA is oxidized to CO_2 at a faster rate than the methyl carbon. Consequently, the methyl carbon remains in the cycle as part of the intermediates for a longer time before being oxidized to CO_2, and would appear in greater concentration than the carboxyl carbon, among the products (amino acids and others) synthesized from cycle intermediates. This relationship would account for the greater specific activity among the amino acids after norleucine-3-C^{14}, which produces acetyl-2-C^{14} CoA, than after norleucine-2-C^{14}, which produces acetyl-1-C^{14} CoA.

The data on the intramolecular labeling of serine, alanine, and glutamate (tables 2 and 3) and the greater specific activities among the amino acids after norleucine-3-C^{14} than after norleucine-2-C^{14}, are consistent with the explanation that the major

pathway in the transfer of carbon to amino acids of casein from norleucine involved the formation of acetyl CoA derived from the C-2 and C-3 carbons of norleucine. This would indicate that the pathway for norleucine metabolism was the same in the intact cow as that established *in vitro* for the rat, namely, deamination [or transamination] of norleucine followed by decarboxylation of apha-keto-caproate and beta-oxidation of valerate to acetyl CoA and propionyl CoA.

SUMMARY

Four lactating cows were injected intravenously with norleucine-3-C^{14} or norleucine-2-C^{14}, and casein was isolated from milk collected three hours later. The specific activities and intramolecular C^{14} distribution were determined for several amino acids recovered from casein samples.

The labeling pattern among the amino acids indicated that norleucine metabolism in the cow involves a pathway that includes deamination, decarboxylation, and beta-oxidation of the resulting valerate.

The effect of administering norleucine to cows at increasing levels for 90 days was studied in terms of the utilization of norleucine carbon for amino acid synthesis. The results indicated that the cows adapted to the norleucine in a manner resulting in greater utilization of norleucine carbon for synthesis of glutamic acid, aspartic acid, serine, glycine, and perhaps alanine.

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Diet and Cholesterolemia

VI. COMPARATIVE EFFECTS OF WHEAT GLUTEN LIPIDS AND SOME OTHER LIPIDS IN PRESENCE OF ADEQUATE AND INADEQUATE DIETARY PROTEIN[1]

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The development of diets for the production of hypercholesterolemia has been reviewed (Portman and Stare, '59). Diets containing cholesterol, cholic acid and hydrogenated coconut oil as the sole source of dietary fat produce severe hypercholesterolemia in the rat (Hegsted et al., '57a; Nath et al., '59b). The severity of the hypercholesterolemia produced with this diet containing 10% of casein can be reduced by increasing the casein content from 10 to 25% of the diet or by substituting wheat gluten for casein (Nath et al., '59a). The effect of a higher casein content of the diet on serum cholesterol concentration can be accounted for by the greater methionine intake of rats consuming a diet containing 25% of casein (Seidel et al., '60), whereas the effect of wheat gluten has been attributed to the presence of lipids associated with the gluten (Nath et al., '59a; de Groot, '59). Replacing part of the saturated fat of the low-casein diet with corn oil has also been found to reduce serum cholesterol concentration (Nath et al., '59b). Effects of combinations of dietary methionine and unsaturated fat on serum cholesterol concentration are reported in this paper together with studies on the nature of the lipid associated with wheat gluten which is responsible for part of the effect of this protein in lowering serum cholesterol concentration.

EXPERIMENTAL

Male weanling rats of the Holtzman strain weighing 50 to 55 gm were used in these experiments. There were 6 rats in each group.

The basal diet contained the following (in per cent): hydrogenated coconut oil, 25; cholesterol, 1; cholic acid, 0.5; salts (Harper, '59), 5; and adequate quantities of all known vitamins (Nath et al., '59b). Sucrose was the source of carbohydrate and all alterations in the diet were compensated for by adjustments in the sucrose content unless otherwise stated.

The animals were weighed weekly and fed the experimental diets ad libitum for three weeks. Food consumption for the entire group was measured. At the end of this period, the food was withdrawn for 15 to 18 hours and then blood was removed by cardiac puncture when the animals were under ether anesthesia. The animals were then killed and their livers removed, weighed and immediately frozen. Serum from each individual animal was analyzed by the method of Henly ('57).

Further details of the method and analytical techniques have been described (Nath et al., '59b).

Procedure for extraction of lipids from wheat gluten. Very little lipid was removed by extraction of wheat gluten with ether or an ether-petroleum ether-acetone mixture for 24 hours in a Soxhlet apparatus. Continuous extraction with absolute ethanol for 24 hours also released only a small amount of lipid. Batch extraction with absolute ethanol or *n*-butanol with continuous stirring at 50 to 55°C for 24

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hours also yielded only small quantities of lipid but after extraction with *n*-butanol in a Soxhlet apparatus for 24 hours, 10 to 11% of the starting material was removed. Further grinding and re-extraction for another 24-hour period with *n*-butanol yielded a total of 12.7% of crude lipids. Batch extraction of wheat gluten with *n*-butanol at 100°C for 24 hours yielded about 10% of lipid consistently; therefore this procedure was adopted for preparing large quantities of extract. From 20 pounds of wheat gluten extracted by this method in a large stainless steel tank, 920 gm of crude lipid was obtained. The butanol was removed by distillation under reduced pressure.

Properties of wheat gluten lipids. The butanol extract of wheat gluten was a brown oily liquid containing 0.8% of nitrogen (by Kjeldahl analysis) and having an iodine number of 89 [Rosenmund-Kuhnhenn method (Williams, '50)]. Biuret- and xanthoproteic tests for protein were negative and qualitative tests for sterols (Lieberman-Burchard and Salkowski tests) showed no detectable amounts of these substances.

The butanol extract was separated into acetone-soluble and acetone-insoluble fractions. Ninety-five per cent of the original extract was soluble in acetone. Saponification of the acetone-soluble fraction yielded fatty acids having an average neutralization equivalent of 318 corresponding to an average chain length of 19 to 20 carbon atoms and an iodine number of 103. The amount of unsaponifiable matter from this fraction was negligible.

The acetone-insoluble fraction was then extracted with diethyl ether and the soluble material turned grayish in color when left at room temperature indicating the presence of phospholipids. The butanol extract thus consisted chiefly of lipids and is referred to hereafter as wheat gluten lipids.

RESULTS

The effects of feeding various fractions of wheat gluten lipids and of substituting butanol-extracted wheat gluten for casein are shown in table 1. The inclusion of 5% of wheat gluten lipids in the diet containing casein caused a marked decrease in serum cholesterol concentration. The acetone-soluble fraction and the fatty acids separated from this fraction, when fed in amounts that would be present in a diet containing 5% of wheat gluten lipids, were equally effective in reducing serum cholesterol concentration. The acetone-insoluble fraction fed at 1% of the diet (equal to the amount in a diet containing 10% of the crude lipids) also reduced the serum cholesterol concentration of the group fed unextracted wheat gluten, which as 25% of the diet supplied 3.2% of lipids, was well below that of the group receiving casein but values for groups fed butanol-extracted wheat gluten were not

TABLE 1

Effect of wheat gluten lipids (butanol extract) on serum cholesterol concentration in the rat

Dietary alterations	Av. daily food intake/ rat	Av. weight gain, 3 weeks	Serum	
			Total cholesterol	Lipid P
	gm	*gm*	*mg/100 ml*	*mg/100 ml*
Basal diet (25% casein)	9.5	102 ± 2[2]	621 ± 130[2]	6.8
Basal diet + 5% WGL[1]	10.6	123 ± 6	307 ± 25	4.9
Basal diet + 4.5% acetone-soluble fraction of WGL	10.2	117 ± 1	297 ± 35	4.8
Basal diet + 3.8% of fatty acids from acetone-soluble fraction	10.6	122 ± 4	302 ± 17	4.8
Basal diet + 1% of acetone-insoluble fraction of WGL	10.9	116 ± 3	440 ± 65	5.7
Unextracted wheat gluten,[3] 25%	6.4	58 ± 1	307 ± 37	5.0
Butanol-extracted wheat gluten,[3] 10%	4.7	3 ± 2	832 ± 108	9.5
Butanol-extracted wheat gluten, 30%	8.9	66 ± 1	707 ± 36	6.8
Butanol-extracted wheat gluten, 67.5%	10.0	116 ± 4	597 ± 70	6.4

[1] Wheat gluten lipids.
[2] Standard error of the mean.
[3] Supplemented with 1.33% of lysine-mono-hydrochloride.

TABLE 2

Comparison of the effects of wheat gluten and various fractions of wheat gluten lipid (butanol extract) on serum cholesterol concentrations of rats fed a hypercholesterolemic diet containing 25% of casein

Dietary additions	Av. daily food intake/ rat	Av. weight gain, 3 weeks	Serum total cholesterol
	gm	*gm*	*mg/100 ml*
None	8.2	109 ± 5[1]	613 ± 83[1]
Acetone-soluble fraction, 1%	8.2	113 ± 5	499 ± 81
Acetone-insoluble fraction I, 1%	8.2	112 ± 5	543 ± 29
Acetone-insoluble fraction II, 1%	7.4	111 ± 3	548 ± 45
Unextracted wheat gluten, 8%	9.0	125 ± 4	519 ± 64

[1] Standard error of the mean.

TABLE 3

Comparison of the effects of corn oil and wheat gluten lipids on the serum cholesterol concentration of rats fed a diet containing 25% of casein

Hydrogenated coconut oil replaced by corn oil or wheat gluten lipids	Av. weight gain, 3 weeks		Serum total cholesterol	
	Corn oil	Wheat gluten lipids	Corn oil	Wheat gluten lipids
%	*gm*	*gm*	*mg/100 ml*	*mg/100 ml*
0	69 ± 10[1]	69 ± 10[1]	622 ± 99[1]	622 ± 99[1]
1	83 ± 8	84 ± 6	481 ± 69	380 ± 27
2	88 ± 5	87 ± 5	554 ± 80	433 ± 104
4	101 ± 8	85 ± 11	353 ± 63	400 ± 52
8	80 ± 3	82 ± 7	339 ± 32	292 ± 9

[1] Standard error of the mean.

lower than those of casein-fed rats. The progressive reduction in serum cholesterol concentration observed with each increase in the quantity of butanol-extracted wheat gluten in the diet was probably due to the higher intake of sulfur-containing amino acids and to the lipids remaining in the wheat gluten after extraction.

The changes in serum lipid phosphorus concentration paralleled changes observed in serum cholesterol concentration.

The effects of adding 1% of the various fractions of wheat gluten lipids or 8% of wheat gluten (supplying 1% of lipid) to the basal hypercholesterolemic diet are shown in table 2. All of the supplements reduced serum cholesterol concentration slightly but none was highly effective when fed in this amount.

A comparison of the effects of several levels of corn oil and wheat gluten lipids is presented in table 3. Both materials reduced serum cholesterol concentration at each level tested.

As hypercholesterolemia is enhanced by reducing the quantity of casein in the diet, an effort was made to magnify the difference between the effect of corn oil and that of wheat gluten lipids by using a diet containing only 10% of casein. The effects of several levels of each lipid were determined using diets containing 10 or 25% of casein and for comparison a stabilized commercial preparation having a high content of polyunsaturated fatty acids[2] and wheat flour lipids[3] were also included. The results are presented graphically in figure 1. The serum cholesterol concentration decreased sharply with addition of up to 1% of any of these unsaturated fats in the diet but tended to level off as the un-

[2] Lenic, a commercial oil containing glycerides of polyunsaturated fatty acids was kindly supplied by Dr. Walter Hoskins, Crooks-Barnes Laboratories, Inc., Wayne, New Jersey, and had the following composition; (mg/gm) tetraenoic, pentaenoic and hexaenoic acid, 100; linolenic acid, 20; linoleic acid, 495; oleic acid, 175; saturated fatty acids, 100; and non-saponifiable matter, 10.

[3] Kindly supplied by Dr. John Andrews, General Mills, Inc., Minneapolis, Minnesota. Extracted from wheat flour at room temperature with ethyl ether and contained 23% of acetone-insoluble fraction and had an iodine value of 123.

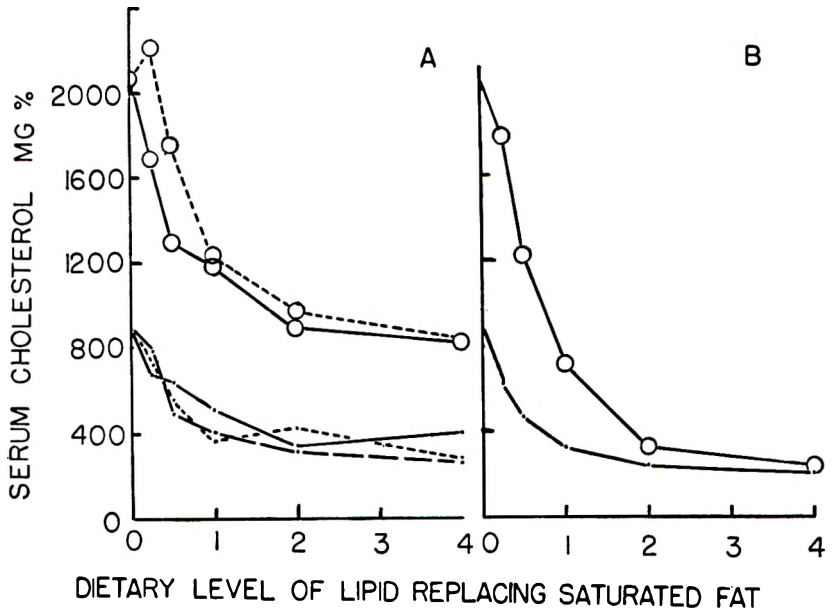

Fig. 1 Effect on serum cholesterol concentration of substituting different levels of other fats for hydrogenated coconut oil in diets containing either 10% (○) or 25% (·) of casein. A, comparison of effects of wheat gluten lipids (—), wheat flour lipids (– –) and corn oil (- - -). B, effect of different levels of highly unsaturated commercial oil (see footnote 2 in text for composition).

saturated fat content of the diet was increased above 1%. Corn oil, wheat gluten lipids, wheat flour lipids and the highly unsaturated oil[4] did not differ significantly in their cholesterol-lowering effects in diets containing 25% of casein. When the diets contained only 10% of casein and less than 1% of unsaturated fat, wheat gluten lipids and the highly unsaturated oil[5] were somewhat more effective than corn oil, but with levels of 1% or higher, the highly unsaturated oil was more effective than either corn oil or wheat gluten lipids.

The greatest effect of additional casein in reducing serum cholesterol concentration was observed when unsaturated fat was omitted from the diet. With diets containing higher amounts of corn oil or wheat gluten lipids, the higher casein content had a smaller but still significant effect upon serum cholesterol concentration. There was no significant effect on serum cholesterol concentration when the casein content of a diet containing 2 or 4% of the highly unsaturated oil[6] was increased from 10% to 25% (fig. 1).

The addition of these unsaturated fats (corn oil, wheat gluten lipids and Lenic[7]), to a diet that was low in protein caused greater deposition of total lipids (an increase from 16.5 to 20.0%) and total cholesterol (an increase from 6.5 to 8%) in the liver. No such effect of unsaturated lipids was observed when the diet was adequate in protein.

Since the serum cholesterol-lowering effect of a high casein intake can be accounted for by the higher intake of sulfur-containing amino acids, the effects of several levels of the highly unsaturated oil[8] on serum cholesterol concentrations of rats fed diets with and without added methionine were determined. The results in table 4 show that the addition of the oil together with methionine or an additional 15% of casein to a diet containing 10% of casein resulted in lower serum choles-

[4] See footnote 2, page 391.
[5] See footnote 2, page 391.
[6] See footnote 2, page 391.
[7] See footnote 2, page 391.
[8] See footnote 2, page 391.

TABLE 4

Combined effect of unsaturated oil[1] and methionine on serum cholesterol concentration of rats fed a hypercholesterolemic diet containing 10% of casein

Dietary additions			Daily food intake/ rat	Av. weight gain, 3 weeks	Serum total cholesterol	Liver[2]	
Lenic[1]	DL-methionine	Casein				Total lipids	Total cholesterol
%	%	%	*gm*	*gm*	*mg/100 ml*	%	%
—	—	—	6.0	35 ± 3	1182 ± 176[3]	16.5	6.79
0.25	—	—	7.3	37 ± 3	831 ± 184		
0.5	—	—	6.8	41 ± 4	547 ± 66		
0.75	—	—	6.9	41 ± 3	455 ± 97	20.9	8.34
2.0	—	—	6.8	37 ± 3	325 ± 29		
4.0	—	—	6.8	42 ± 4	273 ± 18		
—	0.6[4]	—	8.6	69 ± 3	534 ± 37	15.0	6.40
0.25	0.6[4]	—	8.7	67 ± 3	349 ± 20		
0.5	0.6[4]	—	9.1	79 ± 3	284 ± 16		
0.75	0.6[4]	—	9.5	77 ± 4	257 ± 21	23.4	7.20
4.0	0.5	—	7.0	51 ± 6	192 ± 8		
—	—	15	10.3	113 ± 4	758 ± 46	15.0	6.41
0.25	—	15	10.6	124 ± 4	510 ± 45	14.6	5.84

[1] See footnote 2 in text for composition of oil.
[2] Values for pooled samples.
[3] Standard error of the mean.
[4] These diets also contained 0.5% of DL-threonine.

terol concentrations than the addition of casein, methionine or the oil alone.

When the diet contained 10% of casein and 0.6% of DL-methionine, the addition of only 0.75% of the oil to the diet depressed the serum cholesterol concentration to a value (257 ± 21 mg per 100 ml) usually obtained only when 4% or more of the highly unsaturated oil was added to a 10% casein diet containing no additional methionine.

DISCUSSION

The serum cholesterol-lowering effect of replacing dietary saturated fat with unsaturated fat has been repeatedly observed in man (Groen et al., '52; Kinsell et al., '53; Ahrens et al., '54; Beveridge et al., '55) and in experimental animals (Aftergood et al., '57; Hegsted et al., '57a, b; Leveille and Fisher, '58; and Barnes et al., '59). Reviews by Portman and Stare ('59) and Olson and Vester ('60) provide more extensive literature citations. The effects of unsaturated fats on serum lipids in man have been attributed to plant sterols and unidentified factors (Beveridge et al., '57) to total unsaturation (Ahrens et al., '55) and to essential fatty acids (Sinclair, '56).

In contrast with man, conflicting results have been reported for the rat. Swell and Flick ('53) reported higher serum cholesterol concentrations in rats fed soybean oil than in those fed stearate. Klein ('58), Funch et al. ('60) and Nath et al. ('59b) observed slightly higher serum cholesterol concentrations in rats fed diets containing no cholesterol when saturated fat was replaced by unsaturated fat, but Best et al. ('58) and Coleman and Beveridge ('60) could find no significant effect of the type of dietary fat on serum cholesterol concentration under similar conditions. When rats are fed diets containing 1% of cholesterol and a highly saturated fat, however, the replacement of saturated fat in the diet with fat containing polyunsaturated fatty acids has always resulted in a decrease in serum cholesterol concentration (Aftergood et al., '57; de Groot, '59; Hauge and Nicolaysen, '59a, b; and Nath et al., '59b). The only exception we are aware of is the use of tung oil (Hegsted et al., '57a) which contains a large quantity of eleostearic acid, a trienoic fatty acid with conjugated double bonds.

The normal low density β-lipoprotein and cholesterol concentrations of serum are considerably lower in the rat than in man. Feeding cholesterol and hydrogenated fat to the rat, however, results in hypercholesterolemia and an increase in the serum β/α lipoprotein ratio (Seskind et al., '59), a

situation more analogous to that of man (Olson and Vester, '60). Both cholesterol and hydrogenated coconut oil accelerate the appearance of essential fatty acid deficiency in the rat (Holman and Peifer, '60) and definite dermal symptoms of essential fatty acid deficiency are observed in rats fed our basal diet. It appears that the effect of dietary fat containing large amounts of polyunsaturated fatty acids on serum cholesterol concentration in the rat depends to a large extent on the dietary conditions, and that under dietary conditions leading to an essential fatty acid deficiency, a fat rich in polyunsaturated fatty acids may reduce serum cholesterol concentration by alleviating this deficiency. The effect may not be limited to the essential fatty acids, however, since an oil rich in polyunsaturated fatty acids containing 4, 5 and 6 double bonds is even more effective than corn oil when fed as 3 or 4% of the diet. Ahrens et al. ('59) have shown that menhaden oil, a fat low in essential fatty acids but high in polyunsaturated fatty acids, is as effective as corn oil in reducing serum cholesterol concentrations in man, and Worne et al. ('59) have shown the effectiveness in man of an oil containing high amounts of fatty acids with 4, 5 and 6 double bonds.

The reductions in serum cholesterol concentration observed on feeding an acetone-soluble fraction of wheat gluten lipids and the free fatty acids derived from this fraction provide further evidence that unsaturated fatty acids, free of plant sterols, have a marked cholesterol-lowering effect *in rats* on a hypercholesterolemic regimen.[9]

The reduction in serum cholesterol concentration as a result of feeding acetone-insoluble fractions, however, emphasizes that factors other than triglycerides (or fatty acids derived from them) may be important in the regulation of blood cholesterol levels. The cholesterol-lowering effect of this acetone-insoluble fraction is not due to plant sterols, as the qualitative tests for sterols were negative. Furthermore this fraction, though it reduced serum cholesterol concentration, had no effect on the liver cholesterol content, whereas the feeding of β-sitosterol to rats on an identical regimen lowered both serum cholesterol concentration and the deposition of cholesterol in the liver (Nath and Harper, '59). The hypercholesterolemic response of patients ingesting a brain extract has been attributed to the cerebrosides present in brain extract (Jones et al., '56). Compounds of a similar nature are found in wheat gluten lipids and might be responsible, in part, for the cholesterol-lowering effect of the acetone-insoluble fraction of these lipids in the rat. Kinsell et al., ('53) observed a marked decrease in the concentration of cholesterol in the plasma of a patient ingesting phosphatides from mammalian liver and undoubtedly phosphatides are present in the acetone-insoluble fraction of wheat gluten lipids.

The fact that the addition of a highly unsaturated oil and either 0.6% of methionine or 15% of casein to the diet results in lower serum cholesterol concentrations than can be obtained in the absence of unsaturated fat by the addition of up to 2.0% of DL-methionine (Seidel et al., '60) or by raising the casein content of the diet as high as 69.5% (Nath et al., '59a) suggests that the effects of polyunsaturated fatty acids and those of methionine or casein on serum cholesterol concentrations are in some way interrelated. Shapiro and Freedman ('55) showed that methionine was not effective in reducing serum cholesterol concentration in cholesterol-fed rats beyond 40 days unless unsaturated fat was included in the diet.

That the addition of unsaturated fat to a diet containing 10% of casein resulted in an increase in the cholesterol and total lipid content of the liver, whereas this was not observed when the diet contained an adequate amount of protein, also suggests an interrelationship between unsaturated dietary fat and dietary protein.

Leveille et al. ('60) observed that either replacing coconut oil with corn oil or increasing the casein content of the diet reduced the amount of lipid bound to the β-lipoprotein fraction in the serum of chicks. Jones and Huffman ('56) observed that feeding a high-protein diet reduced

[9] Note added in press: Moruzzi, G., M. Martinelli and C. M. Caldarera (Arch. Biochem. Biophys., *91:* 329, 1960) have reported that wheat gluten lipids contain an amount of linoleic acid comparable to that found in corn oil.

the serum concentration of low density lipoproteins in the rat.

Thus the reductions of serum cholesterol concentration in the rat produced by replacing saturated fat in the diet with a fat containing polyunsaturated fatty acids or by increasing the protein or methionine content of the diet appear to be in some way interrelated in that both may affect serum lipoproteins. The possibility of such an interrelationship merits further study.

SUMMARY

The lipids extracted from wheat gluten with *n*-butanol at 100°C, the acetone-soluble fraction of these lipids, the fatty acids separated from this fraction, and the acetone-insoluble fraction all exerted a marked serum cholesterol-lowering effect. Wheat gluten lipids and corn oil were equally effective in reducing serum cholesterol concentration but a commercial oil having a high content of polyunsaturated fatty acids was more effective than either corn oil or wheat gluten lipids.

Serum cholesterol concentrations of rats fed diets containing 10% of protein and 4% of either corn oil or wheat gluten lipids were significantly reduced when the protein content of the diet was raised to 25%. Increasing the protein content of the diet of rats fed 2 to 4% of the highly unsaturated oil had little effect on serum cholesterol concentration, however. Serum cholesterol concentration of rats fed 10% protein diets and smaller amounts of this oil decreased appreciably in response to dietary supplements of casein or methionine.

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The Need for Supplementary Dietary Fat by Breeding Mink Fed Rations Containing Codfish Products[1]

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In the past, horsemeat always formed the predominant ingredient of successful mink rations in those parts of North America where this product could be obtained easily and inexpensively. But now, supplies are too limited for its continued use. Fish, however, is a valuable mink feed ingredient. It is in relatively abundant supply and on the basis of its protein content may serve as a replacement for the meat portion of mink rations. Many types of fresh-water and ocean fish are available, but their successful application to mink feeding is determined particularly by the nature of the fish product used.

Belcher et al. ('58) used precooked fresh-water fish products to replace completely the horsemeat in mink rations. The products, prepared from suckers or smelt, were preserved in a frozen condition until used. Production from females receiving the fish rations, with or without added fat, was equal to or better than that of females receiving the horsemeat rations.

The species of fish used for mink feeding may determine a need, or not, for supplementary fat in the ration. Because the head and viscera (including the liver) are not normally part of the fish products used in mink rations, the distribution of fat in fish, described by the Fisheries Research Board ('52), is particularly relevant to this discussion. Fish such as salmon, herring and pilchard have relatively small livers with little oil in them, but they have an oily muscle tissue. Cod, haddock, hake and halibut, however, have fairly large livers which contain almost all of the fat of the fish. Therefore, the caloric intake of mink fed rations containing a high proportion of codfish products may be limited, especially in view of the low dry matter content of these ingredients.

It is possible that such rations may be calorically inadequate for successful reproduction in the mink. Asdell and Crowell ('35) concluded that energy may be a limiting factor for sexual development in the female rat. When the calorie consumption of female mice was restricted to about two-thirds that of a control group, Ball et al. ('47) found that their fertility was about 10% of the usual value for fully fed females of the same age. Carr et al. ('49) reported that mice remained in anestrus when their caloric intake was restricted by one-half.

The purpose of the work presented herein, was to investigate the effect on reproduction in the mink of hydrogenated animal fat, added as a source of energy to rations containing a high proportion of Atlantic codfish products.

EXPERIMENTAL PROCEDURE

The feeding trial was conducted at the Government Experimental Mink Ranch in Newfoundland. A total of 160 Pastel mink (144 kits and 16 two-year-old adults) were allocated at random to individual pens[2] within the feed lots, three weeks before mating started.

During mating, the animals within each lot were given daily an equal opportunity to mate. A system of "double mating" was adopted whereby a different male was put to a female the day after her first successful mating; the reason for this procedure

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[1] Research conducted in cooperation with the Department of Mines and Resources, Government of Newfoundland, and supported by a grant from the Canada Department of Agriculture for extramural research.

[2] Pens were of conventional design consisting of a wire cage to which was attached a wooden nest box.

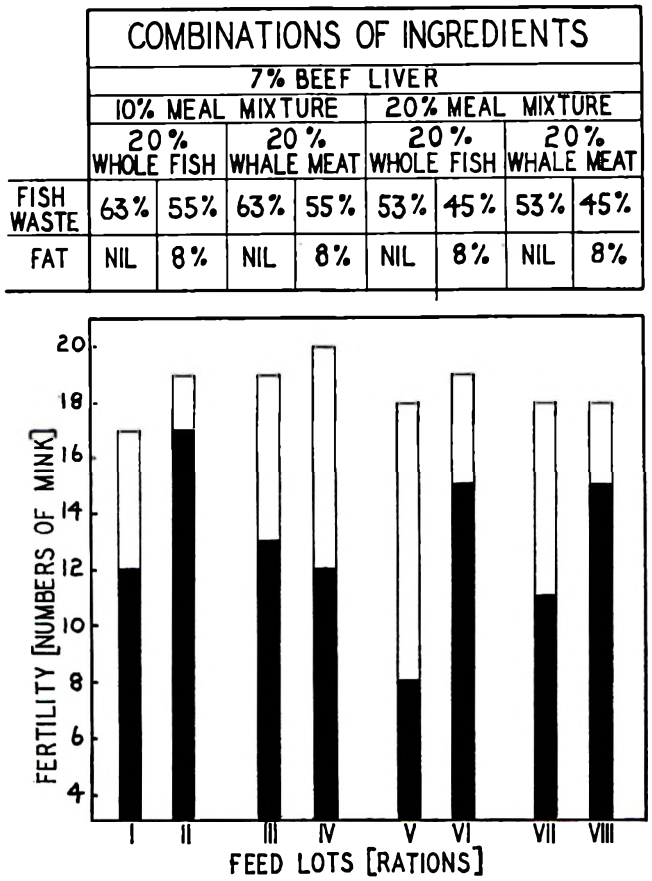

Fig. 1 Comparison of the number of mink whelped with the number mated per feed lot. Each bar represents the number of successfully mated mink and the shaded portion the number of them which whelped. Whole fish refers to whole codfish (head and viscera removed) and fish waste to codfish fillet waste which includes only the bony skeleton, fleshy flank and fillet trimmings, fins and some skin. The fat used was hydrogenated animal fat. The cereal was a commercially prepared mixture intended for use in preparing mink hamburg. (See footnote 3 in text for percentage composition of the mixture.)

has been explained (Friend and Crampton, '61). The experimental rations, fed from the time of allocation, were prepared daily by mixing together the ingredients according to the prescribed formulae[3] (see fig. 1). The marine products were stored frozen until required for ration preparation. The supplementary fat was melted by gentle heat and added, when applicable, to the rest of the ration ingredients during mixing.

The criterion by which the effect of the different rations was judged, was the number of successfully mated mink which whelped in each of the feed lots.[4]

RESULTS AND DISCUSSION

Represented graphically in figure 1 is the fertility of the groups of successfully

[3] Percentage composition of mixture: wheat middlings, 40; ground oat groats, 25; dried brewers' yeast, 15; cane molasses, 5; alfalfa leaf meal, 5; steamed bone meal, 3; iodized salt, 4.6; soybean oil meal, 0.3; vitamin E (Myvamix), 2.0; Aurofac antibiotic, 0.1 (40 gm of chlortetracycline/pound). Gross calories/gram of dry matter of major ration components: liver, 5.7; whale meat, 5.4; whole cod, 4.4; fish waste, 4.5; cereal, 4.5.

[4] Kit numbers averaged 5 per whelped female; this was considered normal for ranched mink in the area.

TABLE 1a

Calculation of chi-square in a 2 × 2 table for the independence of fertility in mink on the level of fat added to the ration[1]

Level of fat added to ration	Fertility			
	Whelped		Not whelped	
	Observed (O)	Expected (E)	Observed (O)	Expected (E)
	%	%	%	%
Nil	61	69.5	39	30.5
8%	78	69.5	22	30.5
Totals	139		61	

Chi-square with (1 d.f.) = $S[(O - E) - 0.5]^2/E = 6.04$.[2]

[1] Snedecor ('56).
[2] Significant at the 2% level of probability.

TABLE 1b

Chi-square values calculated from 2 × 2 tables for the independence of fertility in mink on the level of fat added to "fish-type" rations and to "whale meat-type" rations

Ration composition[1]	Whelped	Calculated χ^2
	%	
"Fish-type"		
Nil fat	57	16.2[2]
8% fat	84	
"Whale meat-type"		
Nil fat	65	0.6
8% fat	71	

[1] "Fish-type" rations refer to those in which 20% of whole codfish (head and viscera removed) were used, as opposed to "whale meat-type" rations in which 20% of whale meat was substituted.
[2] Significant at the 1% level of probability.

mated mink fed similar rations containing either 8% or no supplementary fat. It can be seen from the graph that the addition of fat to the rations fed, generally gave results better than those obtained when supplemented fat was omitted. The difference in fertility was shown to be significant at the 2% level of probability; the statistical analysis is given in table 1A. Because it was considered that the codfish products in the rations were predominantly responsible for a suspected caloric inadequacy, separate statistical analyses of the results from adding fat to the "fish-type" rations and to the "whale meat-type" rations were made.[5] These analyses, as shown in table 1B, confirmed that the overall increase in fertility when fat was added, could be attributed to the difference in fertility, significant at the 1% level of probability, exhibited by the mink fed "fish-type" rations.

It was considered that any essential polyunsaturated fatty acids possibly needed by the mink for satisfactory reproduction were adequately supplied in the rations tested. The difference in fertility between the mink fed the fat supplemented rations and those fed the non-supplemented rations was believed to have been the direct outcome of an increased caloric intake. Rations typical of those used in the trial were calculated to provide about 390 gross calories per mink consuming one-half pound of feed per day. Assuming no change in consumption the addition of 8% of fat to such rations would increase the gross caloric intake, by about 44%, to approximately 560 Cal.

SUMMARY AND CONCLUSIONS

In an experiment in which rations containing a high proportion of codfish products were fed to breeding mink, the addi-

[5] "Fish-type" rations refer to those rations containing 20% of whole codfish (head and viscera removed) as opposed to "whale-meat type" rations containing instead, 20% of whale meat.

tion of 8% of hydrogenated animal fat increased significantly the number of successfully mated mink which whelped. It was concluded that the observed effect was the result of an improved caloric intake.

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Amino Acid Requirements of Children: Minimal Needs of Lysine and Methionine Based on Nitrogen Balance Method

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The present paper is a continuation of the studies in this laboratory on the amino acid requirements of children and is concerned specifically with the minimal needs of lysine and methionine for maintaining a positive nitrogen balance. Studies previously reported have dealt with tryptophan, leucine, and isoleucine (Nakagawa et al., '60, '61). Minimal requirements for lysine and methionine have been determined by other researchers for the male adult, the female adult and the infant. There has been no published data, however, concerning the amino acid requirements for children of school age.

EXPERIMENTAL PROCEDURE AND RESULTS

Minimal needs of lysine and methionine in the absence of cystine were estimated by using the nitrogen balance method. Riboflavin, N^1-methylnicotinamide (N-MNA), creatine and creatinine in the urine and basal metabolism were also determined. Details of the experimental method used were described in previous reports (Nakagawa et al., '60, '61).

Nine healthy 11-year-old boys (table 1) served as experimental subjects, living in the institute under our supervision. For the first three days, these subjects consumed a normal diet including about 12 gm of nitrogen and approximately 1900 Cal. per day. Following the three days of normal diet consumption, the amino acid mixture listed in table 2 and a basal diet derived from cornstarch, corn oil, butter fat, mineral and vitamin mixture[1] were fed.

Experiment 1. The experiment was carried out on 5 subjects for three weeks. Lysine in the amino acid mixture was given at different levels (2.4, zero, 1.2, 0.6 and 1.6 gm) maintaining total nitrogen at a constant level of 12 gm by the substitution of isonitrogenous nonessential amino

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[1] 1.5 mg of riboflavin was given in the present experiment.

TABLE 1

Age, height, weight, basal metabolism and energy intake of subjects

Exp.	Subject	Age	Body height	Body weight	Basal metabolism	Average daily energy intake[1]
		years, months	*cm*	*kg*	*Cal./kg/day*	*Cal./kg*
1	I. U.	10 7	132.1	28.9	39	62
	K. I.	10 7	140.8	27.1	38	84
	O. Z.	10 8	125.5	23.7	39	78
	K. N.	10 8	136.6	28.5	40	64
	I. Y.	11 0	132.0	26.8	37	63
2	T. G.	10 9	133.4	24.8	41	83
	N. S.	11 0	145.5	31.5	33	65
	A. Z.	11 2	139.6	28.7	36	73
	O. S.	11 3	135.1	32.3	31	55

[1] Calories derived from the amino acid mixture are not included.

TABLE 2

Compositions and daily intake of amino acid mixture

Component	Daily intake	Nitrogen content
	gm	*gm*
Essential amino acids		
L-Isoleucine	1.50	0.160
L-Leucine	2.20	0.235
L-Lysine	2.40	0.460
L-Methionine	3.30	0.310
L-Phenylalanine	3.30	0.280
L-Threonine	1.50	0.176
L-Tryptophan	0.40	0.055
L-Valine	2.40	0.287
L-Histidine	0.75	0.203
Nonessential amino acids		
L-Arginine	11.98	3.851
L-Glutamic acid	8.11	0.772
L-Na-Glutamate	12.75	0.954
Glycine	22.92	4.257
Total		12.000

acids. In this experiment, nonprotein nitrogen in the whole blood was also determined.

Results of experiment 1. The results obtained are shown in table 3 and figure 1; average daily energy intakes are given in table 1. Subject I. U. maintained a positive nitrogen balance with 2.4 gm of lysine. When lysine was totally excluded, the nitrogen balance became negative. This subject maintained a positive nitrogen balance even with 1.2 gm of lysine, and the balance became negative with 0.6 gm.

The urinary excretion of creatinine maintained a constant level, but that of creatine increased markedly after consumption of the amino acid mixture described in the previous paper (Nakagawa et al., '61). Urinary riboflavin increased as lysine was excluded, but it did not decrease after the administration of lysine. There seemed to be no relationship between the amount of riboflavin excreted and the nitrogen balance. Urinary N-MNA gradually increased as the lysine was excluded although reaction was delayed in some children. Urinary N-MNA tended to decrease after the administration of lysine. Nonprotein nitrogen in the whole blood maintained a constant level, although Rose et al. ('54) have reported an increase of the nonprotein nitrogen in deficiency periods.

Observations for subjects O. Z. and K. I. were similar to those of I. U. Two subjects, K. N. and I. Y., followed the same pattern as subject I. U., but they could not maintain a positive nitrogen balance with 1.2 gm of lysine. By increasing the intake level to 1.6 gm, a positive balance was attained. Body weight and basal metabolism maintained about a constant level throughout the experiment as well as in the second experiment.

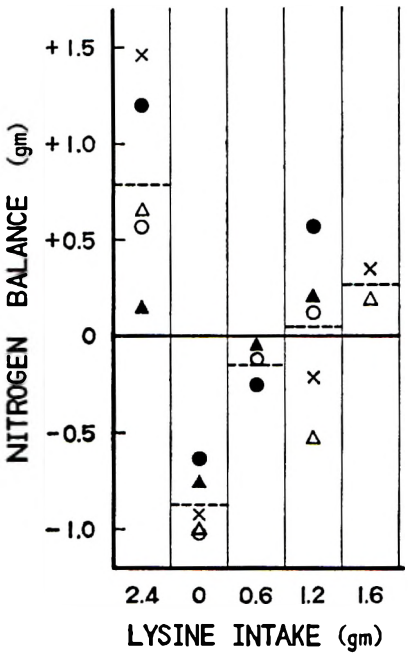

Fig. 1 Lysine intake and nitrogen balance. The dotted line denotes the mean levels of daily nitrogen balance of all subjects at each period; ●, represents the mean nitrogen balance of subject I. U. at each period; ▲, represents that of subject K. I.; △, represents that of subject K. N.; ○, represents that of subject O. Z.; ×, represents that of subject I. Y.

Experiment 2. Four boys served as subjects for 25 days. Methionine in the amino acid mixture from which cystine was excluded was given at different levels (3.3, zero, 1.6, 0.8 and 0.4 gm), maintaining total nitrogen at a constant level of 12 gm. The composition of the nonessential amino acid mixture was modified as shown in table 4. In this experiment free L-methionine in the urine and blood plasma was determined by microbiological assay using *Leuconostoc mesenteroides* P-60.

Results of experiment 2. Results obtained are shown in table 5 and figure 2, and average daily energy intakes in table

TABLE 3

Nitrogen balance and urinary excretion of creatine, creatinine, riboflavin and N-MNA on different levels of lysine intake

Subject	Period	Daily lysine intake	Daily N intake	Average daily N output		Average daily N balance	Average daily urinary excretion			
				Urine	Feces		Creatinine	Creatine	Riboflavin	N-MNA
	days	*gm*	*gm*	*gm*	*gm*	*gm*	*mg*	*mg*	*μg*	*mg*
							722[1]	141[1]		
	5	2.4	12.11	10.36	0.55	+1.20	690	142	538	5.2
I. U.	3	0	12.11	12.08	0.66	−0.63	711	217	641	7.6
	5	1.2	12.21	11.19	0.45	+0.57	682	306	801	9.4
	5	0.6	12.21	11.95	0.51	−0.25	724	384	915	6.7
							528[1]	238[1]		
	5	2.4	12.11	11.03	0.51	+0.57	577	334	489	5.5
O. Z.	3	0	12.11	12.61	0.52	−1.02	614	355	1047	9.2
	5	1.2	12.21	11.65	0.44	+0.12	558	374	1386	10.6
	5	0.6	12.21	11.85	0.48	−0.12	594	367	1170	5.7
							623[1]	67[1]		
	5	2.4	12.11	11.43	0.53	+0.15	697	209	698	4.1
K. I.	3	0	12.11	12.44	0.43	−0.76	629	140	855	5.1
	5	1.2	12.21	11.59	0.42	+0.20	690	194	781	3.8
	4	0.6	12.21	11.65	0.61	−0.05	664	198	1115	5.0
							581[1]	175[1]		
	7	2.4	12.11	10.83	0.63	+0.65	661	239	481	6.8
K. N.	3	0	12.11	12.61	0.51	−1.01	672	191	775	11.1
	4	1.2	12.21	12.22	0.52	−0.53	695	332	804	9.4
	5	1.6	12.21	11.58	0.45	+0.18	704	391	956	7.0
							614[1]	76[1]		
	5	2.4	12.11	10.24	0.41	+1.46	585	209	511	7.9
I. Y.	3	0	12.11	12.65	0.38	−0.92	581	332	710	13.9
	5	1.2	12.21	12.03	0.39	−0.21	597	345	978	17.7
	5	1.6	12.21	11.39	0.47	+0.35	626	384	741	11.8

[1] Average of urinary excretion for three days, when consuming a normal diet.

TABLE 4

Modified composition of nonessential amino acid mixture

Component	Daily intake	Nitrogen content
	gm	*gm*
L-Alanine	0.40	0.063
L-Arginine	12.37	3.977
L-Aspartic acid	8.01	0.843
L-Glutamic acid	12.04	1.144
L-Na-glutamate	14.69	1.099
Glycine	9.35	1.737
L-Proline	0.67	0.081
L-Serine	6.68	0.890
Total		9.834

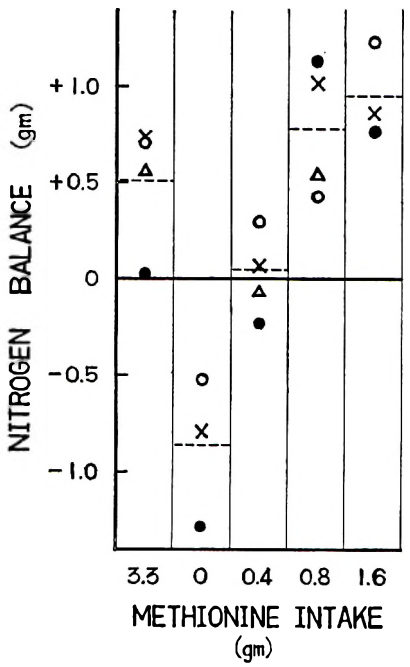

Fig. 2 Methionine intake and nitrogen balance. The dotted line denotes the mean levels of daily nitrogen balance of all subjects at each period; ○, represents the mean nitrogen balance of subject O. S. at each period; ×, represents that of subject T. G.; ●, represents that of subject N. S.; △, represents that of subject A. Z.

1. Subject O. S. maintained a positive nitrogen balance at a level of 3.3 gm of methionine. Following this, when methionine was excluded totally, nitrogen balance became negative. When methionine was added to a level of 1.6 gm, the balance became positive. Thereafter a positive balance was maintained at levels of 0.8 and 0.4 gm.

Urinary excretion of creatinine was maintained at a constant level, but that of creatine increased markedly as described above. The urinary excretion pattern of riboflavin was similar to that observed in the case of lysine. The urinary excretion of N-MNA increased with the exclusion of methionine and decreased after the administration of methionine. Concentration of free methionine in the blood plasma paralleled the amount of methionine intake. Excretion of free methionine in the urine increased when the amino acid mixture including 3.3 gm of methionine was administered, and decreased with the exclusion of methionine.

With subject T. G. similar results were obtained. Subjects N. S. and A. Z. could not maintain a positive nitrogen balance at a level of 0.4 gm of methionine. Other observations were similar to those for subject O. S.

DISCUSSION

The nitrogen balance method is not the best one for determining the amino acid requirements; especially in children, not only a positive nitrogen balance should be maintained, but also allowance for the accumulation of nitrogen for growth and development must be taken into consideration. Although these are the limitations for applying the nitrogen balance method, there is no doubt that it serves as an aid for determining the amino acid requirements for children.

At a level of 2.4 gm of lysine, all subjects maintained a positive nitrogen balance. At a level of 1.2 gm, three subjects were able to maintain a positive nitrogen balance. For the two remaining subjects, nitrogen balance became positive only at a level of 1.6 gm. On the basis of these observations it was estimated that the minimal need of lysine for maintaining a positive nitrogen balance was 1.6 gm or 60 mg per kg for children of school age. The minimal requirements for lysine have been determined by Rose et al. ('55a) at 0.8 gm for the male adult; by Jones et al.[2] at 0.63 gm for the female adult; and by

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

TABLE 5

Nitrogen balance and urinary excretion of creatine, creatinine, riboflavin and N-MNA on different levels of methionine intake

Subject	Period	Daily methionine intake	Daily N intake	Average daily N output		Average daily N balance	Average daily urinary excretion			
				Urine	Feces		Creatinine	Creatine	Riboflavin	N-MNA
	days	*gm*	*gm*	*gm*	*gm*	*gm*	*mg*	*mg*	*μg*	*mg*
							690[1]	17[1]		
	5	3.3	12.11	10.77	0.63	+0.71	829	68	381	6.6
	4	0	12.11	12.09	0.54	−0.52	742	197	1165	10.8
O. S.	4	1.6	12.11	10.39	0.49	+1.23	700	136	913	9.2
	5	0.8	12.11	11.12	0.56	+0.43	745	252	988	7.5
	4	0.4	12.11	11.25	0.56	+0.30	721	221	1200	7.9
							653[1]	54[1]		
	5	3.3	12.11	10.70	0.67	+0.74	714	92	438	5.7
	4	0	12.11	12.41	0.49	−0.79	665	130	804	13.3
T. G.	4	1.6	12.11	10.56	0.69	+0.86	605	120	911	7.7
	5	0.8	12.11	10.38	0.71	+1.02	701	128	692	6.3
	4	0.4	12.11	11.34	0.70	+0.07	644	191	890	6.0
							914[1]	57[1]		
	5	3.3	12.11	11.54	0.54	+0.03	937	81	391	9.4
	4	0	12.11	12.60	0.79	−1.28	866	124	648	11.0
N. S.	4	1.6	12.11	10.54	0.80	+0.77	810	102	687	7.6
	5	0.8	12.11	10.38	0.60	+1.13	878	189	522	5.2
	2	0.4	12.11	11.69	0.65	−0.23	839	228	625	6.3
							748[1]	95[1]		
	5	3.3	12.11	10.84	0.70	+0.57	630	181	883	8.0
A. Z.	5	0.8	12.11	10.76	0.80	+0.55	623	289	791	6.2
	4	0.4	12.11	11.39	0.78	−0.06	630	394	947	5.1

[1] Average of urinary excretion for three days, when consuming a normal diet.

Snyderman et al. ('59) at 105 mg per kg for the infant.

In the methionine study our subjects were able to maintain a positive balance at a level above 0.8 gm, but not always at 0.4 gm. It was estimated that, without cystine the minimal need of methionine for positive nitrogen balance was 0.8 gm or 27 mg per kg. Rose et al. ('55b) determined minimal requirements of methionine as 1.1 gm for the male adult; Swendseid et al. ('56) as 0.55 gm (methionine 0.35 gm + cystine 0.20 gm) for the adult female; and Holt et al. ('59) as 65 mg per kg (with moderate addition of cystine) for the infant.

The values of lysine and methionine per kilogram obtained for children are higher than requirements for the adult male and female, and lower than for the infant.

In the lysine or methionine deficiency periods, the subjects showed symptoms of nervous irritability and failure in appetite, but not to as great a degree as during leucine deficiency (Nakagawa et al., '61). The period of exclusion was only three or 4 days, since the aim was not to learn the effect of the exclusion of lysine or methionine, but to ascertain the negative nitrogen balance. The symptoms might possibly have become more pronounced during prolonged deficiency periods.

Urinary excretion of creatinine maintained almost a constant level, but that of creatine increased markedly after children were fed the amino acid mixture in experiment 1. In experiment 2 in which a smaller amount of glycine was ingested than in previous experiments, the excretion also increased, and did not decrease even with the exclusion of methionine which might be, as well as glycine, one of the precursors of creatine. Further research needs to be done in this area.

According to Pollack et al. ('51), urinary excretion of riboflavin increases or decreases rapidly as the nitrogen balance shifts from a positive to negative balance. In our experiments, the excretion of riboflavin increased with the exclusion of either lysine or methionine, but did not appear to decrease after their administration. A relationship does not appear to exist between the amount of riboflavin excreted and the nitrogen balance. This may be due partly to the large intake of riboflavin, so that the small amount of the riboflavin liberated from labile proteins which include the flavoprotein had little influence on the excretion of riboflavin.

It has been observed that the urinary excretion of N-MNA increased when tryptophan was totally excluded from the amino acid mixture,[3] and an increase of N-MNA excretion was also observed during the lysine and the methionine deficiency period.

CONCLUSIONS

The daily minimal needs for maintaining positive nitrogen balance by children of school age were estimated using five 11-year-old boys for the lysine study and four 11-year-old boys for methionine. The subjects appeared to need 1.6 gm of lysine (60 mg per kg) and 0.8 gm of methionine (27 mg per kg) without cystine for maintaining positive nitrogen balance.

The children excreted a larger amount of creatine after receiving the amino acid mixture, as compared with that after consuming normal diets.

During the periods of lysine or methionine deficiency, the urinary excretion of riboflavin and N^1-methylnicotinamide (N-MNA) appeared to increase, whereas after administration of lysine or methionine, excretion of N-MNA decreased and that of riboflavin did not appear to decrease.

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Hypo- and Hyperthyroidism and the Lipotropism of Diethylstilbestrol in Choline-Deficient Rats[1,2]

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It has been established that diethylstilbestrol produced a lipotropic action in choline-deficient rats which is not due solely to its growth-depressing action (Miller et al., '60). Estrogens are known to influence the activity of the thyroid gland but the exact influence is as yet unknown. Grosvenor and Turner ('59) reported that estradiol benzoate increased the thyroxine secretion rate in female rats. Engbring and Engstrom ('59) showed that estrogen treatment of humans resulted in a decreased utilization of the thyroid hormone. Since estrogens do influence the thyroid gland, this study was made to attempt to determine whether the lipotropic action of diethylstilbestrol (DES) is due to hypo- or hyperthyroidism. Statistical values were determined according to Li ('57).

EXPERIMENTAL

Weanling albino rats (about 3 weeks old) from a stock colony were provided with tap water and experimental diets ad libitum. The basic diet was the same as previously reported (Miller et al., '60) except that a different grade of soy protein[4] was used. Vitamins and diethylstilbestrol (20 ppm) were administered as reported (Miller et al., '58). Thiouracil and thyroid powder[5] were each mixed with the diet at a level of 0.1%. These animals received the experimental diet for 35 days. A commercial laboratory diet,[6] both with and without DES, was given to another group of rats for 45 days. In all experiments, 5 animals of each sex were given each experimental diet.

Rats fed the thyroid preparation were hyperirritable, and their growth rate was depressed. Stidworthy and King ('52) used this preparation for studies of the effects of hyperthyroidism upon vitamin metabolism.

Thiouracil is known to impair the synthesis of the thyroid hormone thereby producing a hypothyroid condition. The rats receiving thiouracil in these studies were obese, indicating that a state of hypothyroidism existed. Thiouracil was also fed at a level of 0.25% of the diet, but many of these animals died before completion of the experimental period.

Livers and adrenals were removed from the animals and stored at −25°C. Livers were extracted, and the percentages of liver lipids were determined as reported by Miller et al. ('58). Adrenals were extracted by the procedure of Duncan ('57). Total sterol was determined by the procedure of Pearson et al. ('54) as further modified by Miller ('60).

RESULTS AND DISCUSSION

The percentages of total liver lipids are shown in table 1. None of the dietary treatments shown in the table produced any changes in the liver lipid content of rats receiving choline-supplemented diets. As previously reported (Miller et al., '58, '60), the addition of DES to a choline-deficient control diet significantly reduced liver lipid accumulation ($F = 154.7$ with 1 and 8 d.f.—males, and $F = 405.8$ with 1 and 8 d.f.—females).

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[3] University Station, P. O. Box 3354.

[4] Drackett Assay Protein C-1, Archer-Daniels-Midland Company, Minneapolis.

[5] Nutritional Biochemicals Corporation, Cleveland.

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

The addition of thiouracil to the choline-deficient control diet also produced a significant decrease in liver lipid accumulation ($F = 87.97$ with 1 and 8 d.f.—males, and $F = 10.83$ with 1 and 8 d.f.—females). The incorporation of DES into this thiouracil-containing diet, however, decreased lipid accumulation below that obtained with the thioracil-containing diet alone ($F = 19.25$ with 1 and 8 d.f.—males, and $F = 6.53$ with 1 and 8 d.f.—females). In fact, the addition of both DES and thiouracil to the choline-deficient diet reduced the lipid accumulation to the level shown by the choline-supplemented controls.

It is probable that thiouracil decreased liver lipid accumulation by a general depression of the metabolic rate of the animals. This would reduce the requirements of essential dietary materials, including choline. The fact that DES still showed its lipotropic action in the presence of thiouracil would indicate that this action of DES is not mediated through an inactivation of the thyroid gland.

The addition of thyroid powder to the choline-deficient control diet had no effect upon liver lipid accumulation (table 1). If DES was added to the choline-deficient diet containing thyroid powder, there was a significant reduction of the liver lipid accumulation when compared with the same diet without DES ($F = 1271.1$ with 1 and 8 d.f.—males, and $F = 12.45$ with 1 and 8 d.f.—females). Therefore, the presence of thyroid powder had no effect upon liver lipid accumulation, and it does not appear that the lipotropic action of DES is mediated through an activation of the thyroid gland.

The percentages of total "sterols" in the livers of rats fed the various diets are listed in table 2. The term "sterols" is used since both cholesterol and compounds structurally related to cholesterol are measured by the analytical method used. The presence of sterols other than cholesterol in human plasma has been shown recently (Greig and Cook, '60). Undoubtedly, cholesterol constitutes the major portion of the sterol fraction present.

An interesting effect of DES upon sterol metabolism was noted. The addition of DES to both choline-supplemented and choline-deficient diets produced an increase in liver "sterols" (table 2). For example, the addition of DES to the choline-supplemented control diet produced a significant increase in liver "sterols" ($F = 27.15$ with 1 and 8 d.f.—males, and $F = 123.5$ with 1 and 8 d.f.—females). It also produced this effect when added to the choline-deficient control diet ($F = 31.68$ with 1 and 8 d.f.—males, and $F = 11.45$ with 1 and 8 d.f.—females). This increase in liver "sterols" by DES also occurred when either thiouracil or thyroid powder was present in the diet and was significant

TABLE 1

Percentages of total liver lipids[1]

Diet	Sex	Choline-supplemented	Choline-deficient
		%	%
Control	♂	19 ± 1.4[2]	52 ± 6.0
	♀	19 ± 1.7	43 ± 2.2
DES, 20 ppm	♂	19 ± 1.7	35 ± 4.6
	♀	18 ± 0.9	32 ± 4.0
Thiouracil, 0.1%	♂	17 ± 1.0	24 ± 3.6
	♀	16 ± 1.0	27 ± 5.0
Thiouracil, 0.1%, plus DES, 20 ppm	♂	16 ± 1.8	16 ± 1.3
	♀	17 ± 1.8	16 ± 1.7
Thyroid powder, 0.1%	♂	18 ± 0.9	47 ± 1.9
	♀	17 ± 1.5	46 ± 8.0
Thyroid powder, 0.1%, plus DES, 20 ppm	♂	16 ± 1.0	28 ± 4.6
	♀	18 ± 0.9	34 ± 2.5

[1] Expressed on a dry-weight basis.
[2] Standard deviations.

TABLE 2

Percentages of total liver "sterols" [1]

Diet	Sex	Choline-supplemented	Choline-deficient
		%	%
Control	♂	0.99 ± 0.05[2]	0.93 ± 0.08
	♀	0.92 ± 0.05	0.77 ± 0.06
DES, 20 ppm	♂	1.83 ± 0.33	1.28 ± 0.15
	♀	1.44 ± 0.10	1.25 ± 0.31
Thiouracil, 0.1%	♂	0.71 ± 0.04	0.95 ± 0.17
	♀	0.64 ± 0.04	0.76 ± 0.12
Thiouracil, 0.1%, plus DES, 20 ppm	♂	2.43 ± 0.75	1.66 ± 0.53
	♀	2.19 ± 0.85	1.66 ± 0.60
Thyroid powder, 0.1%	♂	0.83 ± 0.06	0.91 ± 0.06
	♀	0.86 ± 0.02	0.71 ± 0.09
Thyroid powder, 0.1%, plus DES, 20 ppm	♂	1.12 ± 0.12	1.12 ± 0.14
	♀	1.15 ± 0.13	1.05 ± 0.15

[1] Expressed on a dry-weight basis.

[2] Standard deviations.

TABLE 3

Liver and plasma "sterol" values

Lipid values	Sex	Diet	
		Chow	Chow + 20 ppm DES
Liver "sterol" [1] %	♂	0.78 ± 0.05[2]	1.00 ± 0.05
	♀	0.82 ± 0.06	1.01 ± 0.05
Plasma "sterol," mg/100 ml	♂	43 ± 0.8	30 ± 1.1
	♀	47 ± 6.7	30 ± 2.4

[1] Expressed on a dry-weight basis.

[2] Standard deviations.

when compared with the corresponding diet containing no DES.

This action of DES was investigated in male and female rats receiving a commercial laboratory diet which was adequate with respect to both amino acids and choline. Plasma "sterol" levels were also determined. Blood for these analyses was obtained by heart puncture just prior to the sacrifice of the animals.

The results in table 3 show that DES produced the expected increase in liver "sterols," and this increase was significant ($F = 50.60$ with 1 and 8 d.f.—males, and $F = 31.28$ with 1 and 8 d.f.—females). There was an accompanying decrease in the plasma "sterol" level that was also significant ($F = 49.71$ with 1 and 8 d.f.—males, and $F = 22.23$ with 1 and 8 d.f.—females).

It is known that the liver supplies cholesterol rapidly and in large amounts to the blood (Gould and Cook, '58). If one effect of DES is an increased synthesis of cholesterol in the liver, then it would be expected that there would be a high "sterol" level in the liver and a high, or at least a normal, "sterol" level in the plasma. The production of a low plasma "sterol" level by DES, as obtained in this investigation, would require an accelerated removal of plasma "sterols." It has been reported that both hexestrol and DES cause a large reduction in the biological half-life of plasma cholesterol (Boyd, '60).

If DES does not increase cholesterol synthesis in the liver, then the high liver "sterol" level and the low plasma "sterol" level could be explained by an impaired transport of cholesterol from the liver to

the blood and an accelerated removal of plasma cholesterol. An effect of DES upon lipid transport has already been noted in its lipotropic action in choline deficiency (Miller et al., '58, '60).

The percentages of adrenal "sterols" are shown in table 4. The presence of DES in the various diets significantly reduced adrenal "sterol" levels when compared with corresponding diets containing no DES. This was noted in earlier choline-deficiency studies (Miller et al., '60). Barnett and Teague ('58) have reported that DES caused a stimulation of activity in the adrenal cortex of the rat which was probably caused by the release of ACTH. Both an activation of the adrenal cortex and a lipotropic action in choline deficiency by DES are difficult to correlate, particularly when it has been suggested that nutritional fatty livers may be caused by a prolonged hyperfunction of the adrenal gland (Le Breton, '56). It has recently been reported that estrogenic treatment of humans produced an increase in plasma cortisol but that this was an increase in protein-bound cortisol which is thought to be biologically inactive (Peterson et al., '60). Additional evidence for this was noted in the report that the failure of humans treated with estrogens to show physiological evidence of an excess of cortisol was due, in part, to a failure of tissue levels of cortisol to rise to the same degree as plasma levels (Robertson et al., '59). Consequently, DES may not produce as severe an effect on the adrenal cortex as its action upon adrenal "sterol" levels indicates.

The protein used in these experiments was soybean protein which is low in methionine. This type of protein accentuates choline deficiency because methionine can serve as a source of methyl groups in the biosynthesis of choline. This also means, however, that methionine was a limiting factor in the diet and that any changes in the dietary protein could be expected to influence experimental results. A comparison of some of the results of our past and present work has shown this to be true. For example, simply by changing the grade of soybean protein, it was noted that the accumulation of liver lipids during choline deficiency was not as extensive as previously reported (Miller et al., '58, '60), and DES did not increase the lipid content of livers in choline-supplemented rats as previously reported (Miller et al., '60).

TABLE 4

Percentages of total adrenal "sterols" [1]

Diet	Sex	Choline-supplemented	Choline-deficient
		%	%
Control	♂	5.6 ± 0.5[2]	5.9 ± 0.5
	♀	5.7 ± 0.5	5.9 ± 0.6
DES, 20 ppm	♂	2.3 ± 0.3	2.0 ± 0.2
	♀	2.3 ± 0.4	2.3 ± 0.3
Thiouracil, 0.1%	♂	5.3 ± 0.7	5.3 ± 1.1
	♀	5.3 ± 0.6	6.0 ± 1.0
Thiouracil, 0.1%, plus DES, 20 ppm	♂	3.2 ± 0.9	3.0 ± 0.7
	♀	2.8 ± 1.2	2.6 ± 0.7
Thyroid powder, 0.1%	♂	6.2 ± 0.4	5.5 ± 0.6
	♀	5.5 ± 0.4	5.8 ± 0.7
Thyroid powder, 0.1%, plus DES, 20 ppm	♂	2.9 ± 0.5	2.8 ± 0.5
	♀	2.0 ± 0.4	2.9 ± 0.4
Commercial diet	♂	3.3 ± 0.4	—
	♀	4.0 ± 0.7	—
Commercial diet, plus DES, 20 ppm	♂	1.2 ± 0.1	—
	♀	0.7 ± 0.2	—

[1] Expressed as percentage of fresh tissue.
[2] Standard deviations.

SUMMARY

The lipotropic action of dietary diethylstilbestrol (DES) in male and female rats receiving a choline-deficient and low-methionine diet was not influenced either by the hypothyroidism produced by dietary thiouracil or the hyperthyroidism produced by a dietary thyroid preparation. Dietary thiouracil caused a decrease in liver lipid accumulation in the absence of dietary DES, but the thyroid preparation produced no change in liver lipid accumulation.

Dietary DES caused an increase in total liver "sterols" when added to both choline-deficient and choline-supplemented diets. This also occurred when either thiouracil or the thyroid preparation was present in the diet. When DES was added to a commercial laboratory diet, not only did the rats show increased liver "sterol" levels but, in addition, showed decreased plasma "sterol" levels.

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Problems in the Prediction of Protein Values of Diets: The Use of Food Composition Tables

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Until recently the only means of assessing the protein value of human diets has been by biological assay of net dietary protein value which is equal to net protein utilization operative ($NPU_{(op)}$) multiplied by the protein content of the diet, and is a measure of utilizable protein.

Miller and Payne ('61) proposed an equation for the prediction of protein values from chemical data:

$$\text{NDpCals \%} = \text{"score"} \times P\left(\frac{54 - P}{54 - P_m}\right) \quad (1)$$

where NDpCals % = net dietary protein calories % (Platt and Miller, '59); P = protein calories %; P_m = 400/"score" = protein calories % required for maintenance. "Score" = protein score based on chemical data (FAO, '57a). This equation which was derived from biological data, may also be obtained theoretically as follows. Miller and Bender ('55) determine NPU from the expression:

$$NPU = \frac{B - (B_k - I_k)}{I}$$

As I_k (nitrogen intake) is zero on a protein free diet, NPU may be defined as:

$$NPU = \frac{B - B_k}{I} = \frac{(B - B_o) - (B_k - B_o)}{I} \quad (2)$$

where B_o = carcass N of animals initially; B = carcass N of animals fed protein diet; B_k = carcass N of animals fed protein-free diet; I = N intake of animals fed protein diet.

Since endogenous nitrogen is a function of body size, the above definition is strictly true only for infinitely short periods of time. An alternative definition of NPU could be in terms of increments of N gain per increment of N intake in calculus notation.

"I" may be considered to be diverted into three metabolic pathways: N used for maintenance (I_m); for growth (I_g) and for energy purposes (I_e);

$$I = I_m + I_g + I_e \quad (3)$$

For this purpose I_e does not include residues from the inefficient use of I_m and I_g but is simply that amount of N oxidized inevitably and directly for energy purposes and which does not enter the anabolic pathway. This is shown (Hamilton, '39) by the decrease in NPU at high protein concentrations which is nevertheless accompanied by an increase in the rate of growth, indicating competition for protein between growth and energy purposes.

Let us assume that the efficiency of utilization of I_g is the same as for I_m and equal to *b*. This assumption is difficult to establish because of the inevitable existence of I_e which becomes important under conditions of growth. Evidence for differences of protein utilization (Mitchell, '59) or amino acid requirements (Osborne and Mendel, '14) between adult and growing rats does not invalidate such an hypothesis for this reason. It would seem likely, however, that the amino acid pattern required for the deposition of new tissue is similar to that for tissue maintenance.

Thus

$$b = \frac{B_o - B_k}{I_m} \text{ and } b = \frac{B - B_o}{I_g}$$

Therefore

$$B_o - B_k = bI_m \quad (4)$$

Similarly

$$B - B_o = bI_g \quad (5)$$

NPU may be defined as "standardized" or "operative" according to whether the measurements are made below or above maintenance (Miller and Payne, '61). NPU standardized ($NPU_{(st)}$) is constant at low

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levels of protein intake and is directly related to protein score. $NPU_{(op)}$ may be obtained from equations (2), (4), and (5):

$$NPU_{(op)} = b\,\frac{(I_m + I_g)}{I}$$

From equation (3):

$$I_m + I_g = I - I_e$$

Therefore

$$NPU_{(op)} = b \cdot \left(1 - \frac{I_e}{I}\right) \qquad (6)$$

As the concentration of protein in the diet is increased, an increasing proportion of it is diverted from the anabolic pathway and oxidized. This is supported by the observation that the biological value of protein decreases with increase in protein in the diet (Platt and Miller, '58; Miller and Payne, '61; Henry and Kon, '57; Forbes et al., '58); also by the protein-sparing effect of additional carbohydrate (Munro, '51). For the present purposes it is assumed that the fraction of the food protein diverted for energy is directly proportional to the concentration of protein calories in the diet. Thus:

$$\frac{I_e}{I} = kP \qquad (7)$$

where P is the percentage of protein calories in the diet. This hypothesis is based on a consideration of the principles of mass law; in this case, the rates of oxidation of amino acids and glucose are simply proportional to their concentrations at the cell. The work of Barnes et al. ('46) shows that "waste energy" derived from protein is substantially proportional to the concentration of protein in the diet.

From equation (6) and (7):

$$NPU_{(op)} = b\,(1 - kP) \qquad (8)$$

By definition $NPU_{(op)} = NPU_{(st)}$ when $P = P_m$; thus from equation 8:

$$NPU_{(st)} = b\,(1 - kP_m)$$

$$b = \frac{NPU_{(st)}}{1 - kP_m} \qquad (9)$$

From equations (8) and (9):

$$NPU_{(op)} = \frac{NPU_{(st)}}{1 - kP_m} \cdot (1 - kP)$$

$$= NPU_{(st)} \cdot \left(\frac{\frac{1}{k} - P}{\frac{1}{k} - P_m}\right)$$

$$NDpCals\ \% = NPU_{(op)}\,P$$

$$= NPU_{(st)} \cdot \left(\frac{\frac{1}{k} - P}{\frac{1}{k} - P_m}\right) \cdot P \qquad (10)$$

This equation may be compared directly with equation (1) when $NPU_{(st)}$ is equated to "score," and 1/k to 54. The theoretical derivation of this equation supports its application to proteins in general, and for this purpose we have constructed a nomograph (fig. 1) showing the relationship between score, P, and NDpCals %.

Given an estimate of "score" from chemical data, it is possible to read off NDpCals %. Below maintenance (i.e., the region on the nomograph below the hyperbola NDpCals % = 4) NPU is constant, i.e., $NPU_{(op)} = NPU_{(st)}$ = "score." Above maintenance $NPU_{(op)}$ decreases with increase in protein concentration; the extent of this decrease may be obtained from the nomograph by laying a ruler from that point on the hyperbola NDpCals % = 4 corresponding to the "score" in question, to the point marked X on the protein-concentration axis. The lowest possible protein "score," or alternatively, the lowest concentration of protein in a mixture required to achieve any given NDpCals %, may also be seen on the nomograph. This paper describes its use for the prediction of the protein values of human diets.

METHODS

NPU was determined on rats by the method of Miller and Bender ('55). For reasons outlined above we distinguish between the $NPU_{(st)}$ measured at the lowest protein level sufficient to maintain nitrogen equilibrium in diets adequate in all other respects and $NPU_{(op)}$ measured under any other conditions in accordance with Platt and Miller ('59).

The human diets were prepared according to national food survey data, and meals or dishes according to traditional recipes; they were freeze-dried, and fed unmodified. Metabolizable energy was determined by the method of Miller and Payne ('59) and the percentage of energy supplied by protein was calculated from:

$$\frac{25 \times \text{N per cent}}{\text{metabolizable energy per gm}}$$

The calculated protein values of the foods were estimated using the original survey data and recipes. The protein and calorie contents of the ingredients were taken from tables given by Platt ('45) and McCance and Widdowson ('60). The

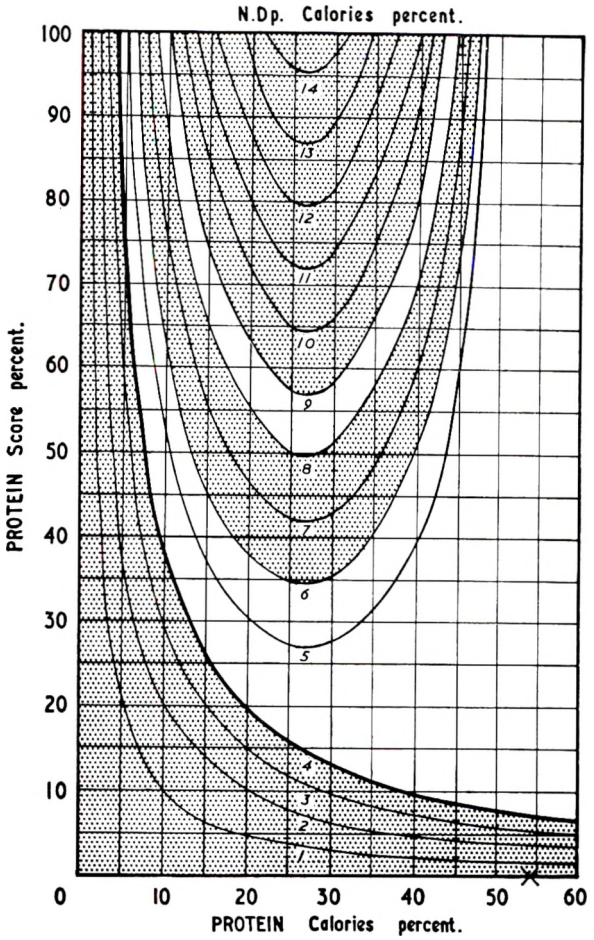

Fig. 1 Nomograph for the prediction of the protein value of diets. The parabolas are lines of equal NDpCals %.

amino acid composition of the proteins was taken from Orr and Watt ('57). The protein "score" was calculated using the method of FAO ('57). Values for NDpCals % were read off the nomograph knowing the protein calories percentage and "score"; for example, a mixture containing 15% of protein calories with a "score" of 65% may be seen to have a NDpCals of 8%.

RESULTS

A comparison is made in table 1 between protein values calculated using food tables only and those determined directly by rat assay. The examples chosen have been derived from a wide range of localities, and have been constructed from a representative number of staples. The degree of complexity of many is high and almost the complete range of protein values is covered (zero to 14.7 NDpCals %) (Miller and Payne, '61).

As expected $NPU_{(op)}$ may not be equated directly to "score" and the difference between these values clearly depends upon protein concentration. When allowance has been made for this factor, the calculated values are in good agreement with the results of bioassay, as will be seen from a comparison of the values given by each of the two methods expressed in the form of NDpCals %. Two thirds of the results agree within 0.5 NDp units and 95% within 1.2 units.

In the second series of results presented in table 2, a similar comparison is made

TABLE 1

A comparison of the protein values of some human diets as determined by rat assay and as calculated from food tables

Origin	Staple[1]	Additional effective sources of protein[2]	Total no. of components	Observed values			Calculated values[4]		
				$NPU_{(op)}$	Protein Calories	NDpCals[3]	Score	Protein Calories	NDpCals
				%	*% total Calories*	%	%	*% total Calories*	%
Gambia	Cassava	Pulses	4	45	2.8	1.3	53	2.1	1.1
Papua	Sago	Fish	3	75	3.5	2.6 ± 0.2	74	4.1	3.0
Gambia	Cassava	Pulses	6	40	7.5	3.0 ± 0.1	51	7.4	3.8
Jamaica	Sugar	Cornmeal	3	66	4.9	3.2 ± 0.1	45	4.3	2.0
Gambia	Cassava	Fish	5	65	6.1	4.0 ± 0.3	64	8.9	5.3
Britain	Potato	—	3	51	9.3	4.7 ± 0.0	55	9.6	5.0
E. Pakistan	Rice	Pulses, milk	14	59	9.2	5.4 ± 0.2	69	9.3	5.9
Jamaica	Maize	Fish	4	60	10.0	6.0 ± 0.2	58	14.0	6.8
Britain	Wheat	Cheese	6	73	9.4	6.9 ± 0.3	76	10.7	7.1
Nigeria	Sorghum	Pulses, fish	12	58	12.5	7.3 ± 0.4	72	12.5	7.7
Gambia	Rice	Pulses, fish	3	63	11.2	7.1	68	12.2	7.2
Gambia	Maize	Pulses, fish	6	57	13.9	7.9	68	14.2	8.0
Britain	Wheat	Cheese	4	51	15.7	8.0 ± 0.3	73	13.1	8.0
Britain	Wheat	Milk, meat, eggs	11	63	13.1	8.2 ± 0.2	80	11.5	7.9
Persia	Wheat	Meat, eggs, milk	11	55	15.0	8.3 ± 0.3	76	13.0	8.3
Nigeria	Sorghum	Milk, fish	13	63	14.0	8.8 ± 0.3	76	15.1	9.0
Britain	Potato	Fish	2	47	18.7	8.8 ± 0.0	70	18.7	9.6
Turkey	Wheat	Meat, pulses	11	59	15.7	9.2 ± 0.2	71	15.3	8.7
Gambia	Rice	Pulses, fish	6	37	25.0	9.2	67	19.0	9.3
Britain	Potato	Meat	2	55	17.2	9.4 ± 0.0	74	16.5	9.4
Britain	Potato	Eggs	2	72	14.0	10.1	100	14.0	11.2
Britain	Wheat	Fish, milk	5	47	23.0	10.8 ± 0.2	80	23.0	11.5
Britain	Wheat	Milk	5	44	29.0	12.8 ± 0.2	80	29.0	11.8

[1] Chief source of calories.
[2] Foods (excluding the staple) contributing more than 20% of the protein in the diet.
[3] The figures given are means, and the limits are standard errors.
[4] Food composition tables used were McCance and Widdowson ('60); Orr and Watt ('57); and Platt ('45).

TABLE 2

Calculated and observed protein values for a Ghanaian diet with and without various supplements

Supplement[1]	Observed values			Calculated values		
	$NPU_{(op)}$	Protein Calories	NDpCals	Score	Protein Calories	NDpCals
	%	*% total Calories*	%	%	*% total Calories*	%
None	53	9.7	5.2 ± 0.1	64	9.7	5.7
0.25% DL-Methionine	55	9.7	5.3 ± 0.3			
6% Whole dried egg	65	11.2	7.3 ± 0.0	78	12.0	8.0
10% Dried meat	51	17.1	8.7 ± 0.1	72	17.3	9.3
10% Leaf protein[2]	41	14.4	5.9 ± 0.4	55	14.8	6.8
20% Leaf protein	40	19.0	7.6 ± 0.7	51	18.9	7.4
40% Leaf protein	25	28.4	7.1 ± 1.2	47	26.5	7.4
10% Leaf protein + 0.1% DL-Methionine	53	14.4	7.6 ± 0.2	61	14.8	7.4
20% Leaf protein + 0.2% DL-Methionine	—	19.0	— —	62	18.9	8.7
40% Leaf protein + 0.4% DL-Methionine	35	28.4	9.9 ± 0.5	62	26.5	9.6

[1] The supplements were added in the percentages indicated to the dry weight of the diet.
[2] Supplied by N. W. Pirie, Rothamstead Experimental Station, Harpenden, Herts. This material had a protein content of 56%, 4.9 Cal./gm, and a $NPU_{(st)} = 42\%$ rising to 62% when supplemented with methionine.

using a recipe sent to us from Ghana.[1] The meal consisted of:

Fresh cassava	109
Dried fish	7
Tomatoes	2
Garden eggs	7
Onions	0.5
Pepper	0.5
Red palm oil	6

Analytical data was not available for Ghanaian dried fish; the material used was therefore assayed and found to contain 63% of protein and 2.7 Cals/gm. The $NPU_{(st)}$ was 70%, increasing to 78% when supplemented with methionine; thus, it was assumed to have a total sulphur amino acid content of 0.19 gm/gmN (i.e., 70% of the value given by FAO for the provisional pattern).

The meal alone and with various supplements was scored on the basis of its content of sulphur amino acids, and the predicted results are again in good agreement with bioassay.

DISCUSSION

Methods for the prediction of the protein values of diets must be considered in relation to the accuracy which can be obtained using the data available, and to the accuracy required to assess or prescribe diets with reference to requirements. Recommended allowances for man are given in figure 2: these have been calculated from the figures given by FAO ('57a, b), and should be considered in relation to (a) the discussion by the committees of this organization of "minimum," "average," and "optimum" requirements; (b) the allowance of "an arbitrary increment of 50% over average minimum requirements;" and (c) the minimum requirements proposed by other authorities, e.g. N.R.C. ('59) and Hegsted ('59).

In those areas of the world where the assessment of the protein values of diets is most important, the survey data available is frequently scarce and unreliable. Factors affecting the validity of such data include (a) seasonal and geographical variations; (b) the consumption of spaced meals by man; and (c) the distribution of food within the family (Platt et al., '61).

Food composition tables have been compiled for application to surveys and necessarily consist of "representative values" chosen from a range for each food (Platt, '45) and such values differ according to the authority. It is for this reason that the calculated values for protein content do not agree closely in all cases with those determined by direct analysis. The range of reported values for amino acid composi-

[1] Supplied by Miss Pauline Whitby.

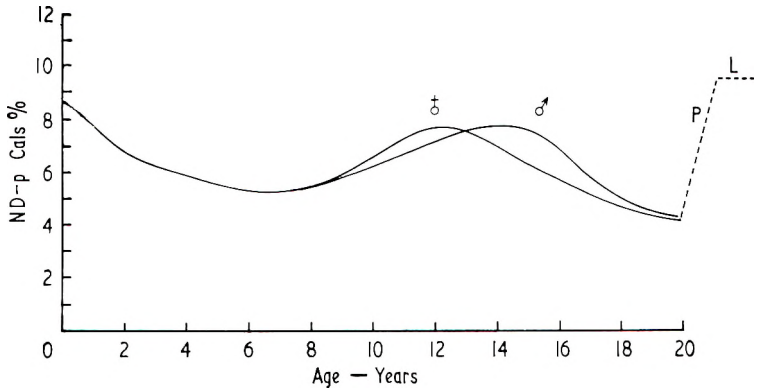

Fig. 2 Safe practical allowances of protein for various human subjects expressed as NDpCals %. P indicates pregnancy; L, lactation.

tion of foods is many times wider than that for other nutrients (Orr and Watt, '57); and there is a need for a table of representative values in which due account is taken of (a) the method of hydrolysis; (b) the method of analysis; (c) the number of determinations; (d) the biological availability of the amino acids; and (e) the agreement of protein scores based on the selected values with $NPU_{(st)}$. It has not been possible in this paper to take these factors into account.

In the present work a deliberate attempt was made to evaluate the limit of all these variants by calculating the protein values of human diets by using food tables alone. Despite the many sources of possible error the predicted results show good agreement with those determined by rat assay. It would appear that this technique is sufficiently accurate for the evaluation of dietary data based on records of group intake, but it may be that for precise nutritional investigations on individual human and animal subjects direct determination of some of the more important amino acids would be advantageous (Miller and Payne, '61). The results in table 2 indicate the value of the technique for prescribing supplements required for the improvement of the protein value of a diet to any given recommended level.

In the introduction to this paper a theory is proposed substantiating the equation derived empirically from biological data. Evidence in support of this theory is given in this paper by demonstrating its general applicability to a wide range of protein mixtures and concentrations. It should be stressed that the equations would not apply in cases of inadequate caloric intake where increased amounts of protein may be used for energy purposes (Allison, '58). Equations dealing with this effect will be given in a subsequent communication.

SUMMARY

1. A method for the prediction of the protein values of diets was presented and shown to give values which are in good agreement with those obtained by bioassay.

2. A theoretical basis was proposed for the equations used.

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The Influence of Chain Length of the Saturated Fatty Acids on Their Effect on Serum Cholesterol Concentration in Man[1]

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Studies on man under controlled conditions in this laboratory have shown that the average response of the cholesterol concentration in the blood to alterations in the fats in the diet can be predicted with reasonable accuracy from information on the proportions of saturated and polyunsaturated fatty acids in the diet before and after the change. (Keys et al., '57, a, b, '59; Anderson et al., '57). The prediction equation derived from these studies has given satisfactory agreement with experimental data obtained by others (Ahrens et al., '57; Malmros and Wigand, '57; Turpeinen et al., '60).

Data used for these purposes involve the specification of the fatty acids in the diet in units of weight or proportion of total calories in the diet calculated from the weights. In general, the data used in deriving the prediction equation and testing its accuracy were obtained from experiments in which there were no great differences in the average chain lengths of the carbon atoms in the fatty acid molecules, namely, the weights of the fats were at least roughly proportional to the number of fatty acid molecules. It is conceivable, however, that the effects of the fatty acids on the cholesterol level are more strictly proportional to the number of molecules than to the gross weight of the fatty acids. If this were the case, discrepancies would appear when comparison is made of the effects of fats differing considerably in the average chain lengths of the contained fatty acids.

Ahrens et al. ('57), from observations with diets containing butter and coconut oil, concluded that saturated fatty acids with short carbon chains cause higher cholesterol concentrations in the serum than equal weights of long-chain fatty acids. Our own experiments with butyric acid in the diet indicated that, at the highest level it occurs in natural diets, this fatty acid has no remarkable effect (Keys, '58). It is possible, however, that fatty acids with chains of 6 to 14 carbon atoms have different effects than fatty acids with shorter or longer carbon chains. This would be important because some vegetable food oils (coconut, palm kernel) are very rich in lauric acid, which has 12 carbons in the chain.

Accordingly, controlled dietary experiments on man were made in order to evaluate the effect on serum cholesterol concentration of equal amounts of two fat mixtures of similar composition in terms of saturated, monoene and polyene fatty acids but differing in the chain length of the saturated acids. The results of two experiments are presented here. They indicate that the saturated fatty acids with 12 to 14 carbon atoms in the chain have a slightly greater serum cholesterol-raising effect in man than equal weights of the saturated fatty acids with 16 to 18 carbon atoms.

SUBJECTS AND METHODS

The subjects of experiment M were 27 physically healthy schizophrenic men 38 to 65 years old. They were divided into two groups matched with respect to age, relative body weight and psychiatric diag-

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[1] This work was supported in part by a research grant from the U. S. Public Health Service, recommended by the National Advisory Heart Council, and by grants from the American Heart Association, New York, the National Dairy Council, Chicago, and the Minnesota Heart Association.

nosis. After the men had been standardized for several weeks with the usual house diet, the two experimental diets were given in a switchback or reversal plan. Diet 12C was given to one group of men and diet 18C to the other group for three weeks. Then the diets were reversed and the feeding continued three weeks longer. A single basic low-fat diet was used to which were added 100 gm of either a 12-C supplement or an 18-C supplement. The 12-C supplement consisted of 56% of coconut oil and 44% of olive oil; the 18-C supplement contained 98% of oleostock and 2% of safflower oil. These two supplements were designed to be equal in polyene fatty acids, in monoene fatty acids and in total amount of saturated fatty acids but to differ markedly in the relative amounts of short-chain and long-chain saturated fatty acids. The supplements were analyzed by gas liquid chromatography using a Craig polyester column.

The composition of the standard daily serving, including the fat supplement, computed from food tables, was protein, 99 gm; fat, 138 gm; carbohydrate, 390 gm; and, Calories, 3195. Individual men were given extra bread and jelly if necessary to keep their body weights constant. Records were kept of foods eaten in addition to the standard serving and of quantities of food rejected. The dietary records indicated a mean total fat intake of 133 gm when consuming diet 12C and of 136 gm for diet 18C. This included an intake of 98 gm of 12-C supplement and 99 gm of 18-C supplement. These figures show that the intake of basic diet fat was nearly constant, 35 gm for diet 12C and 37 gm with diet 18C. Mean caloric intake was 3085 for diet 12C and 3058 for diet 18C.

The amounts of fatty acids in the quantities of supplements eaten are given in table 1. In the columns headed "Excess" are given the effective differences between diets. When receiving diet 12C the men ate a daily excess of about 44 gm of glycerides of saturated fatty acids ranging from 6 to 14 carbon atoms in chain length, the preponderance being of the 12-carbon member, lauric acid. When consuming diet 18C the excess was about 43 gm of roughly equal parts of glycerides of stearic

TABLE 1

Fatty acids, as glycerides, in the two supplements actually eaten in experiment M (gm/man/day)

Fatty acids[1]	12C Supplement	18C Supplement	Excess	
			12C	18C
8:0	4	0	4	0
10:0	4	0	4	0
12:0	29	0	29	0
14:0	10	3	7	0
15:0	0	1	0	1
16:0	9	26	0	17
17:0	0	2	0	2
18:0	2	25	0	23
14:1	0	1	0	1
16:1	1	4	0	3
17:1[2]	0	1	0	1
18:1	33	29	4	0
18:2	6	6	0	0
18:3	0	2	0	2
Saturated 8 to 14	47	3	44	0
Saturated 15 to 18	11	54	0	43
Total saturated	58	57	1	0
Total monoene	34	34	0	0
Total polyene	6	8	0	2
Total glycerides	98	99	0	1

[1] The first figure represents the number of carbon atoms and the second the number of double bonds.

[2] Tentative identification by analogy with the findings of Woodford and van Gent ('60).

and palmitic acids, the 18- and 16-carbon chain saturated fatty acids.

The subjects and diets of experiment AB were as nearly as possible like those of the earlier experiment. The 23 men ranged from 37 to 59 years of age and included several of those used before. They were divided into 4 matched groups using serum cholesterol level in addition to the criteria used previously. In period 1 all the men ate the same diet (controlled house diet). In periods two and three the first two groups were given diets 12C and 18C alternately while the other groups were eating other experimental diets. Later in periods 4 and 5 the last 2 groups were given diets 12C and 18C in the same switchback plan. At the end every man had eaten both diets.

The composition of the fats used, determined by gas liquid chromatography, are given in table 2. In order to match the composition of the former supplements the new 12-C supplement consisted of 58% of coconut oil and 42% of olive oil, and the new 18-C supplement consisted of 54% of oleostearine, 40% of oleostock and 6% of safflower oil, these changes in proportions being necessary because the new supplies of fats were different in composition from the original ones. Oleostock is the filtered fat from beef internal organs with some admixture of fat trimmings from the carcass. Oleostock is separated by crystallization and pressing into a solid, oleostearine, and a liquid, oleo oil.

A standard serving of each diet was collected for 7 days at a time on two occasions and analyzed by methods which were conventional except for the fat determination which was done by a modification of the method described for feces by Söderhjelm and Söderhjelm ('49). In this method the homogenate is suspended in 60% ethanol with dilute HCl and the fat is extracted into a mixture of equal parts of ethyl ether and petroleum ether. This method has been found to give as complete extraction of fat as any other tried. After adjusting for extras, rejections and the quantity of 18-C supplement found adhering to the dish in which it was served (3.6 gm per man per day), the mean amounts of nutrients consumed with diets 12C and 18C were 77 and 81 gm of protein, 146 and 142 gm of fat, and 2890

TABLE 2

Gas chromotography analysis of the oils and fats and of the 12C and 18C supplements of experiment AB (gm of glycerides/100 gm of total glycerides)

Fatty acids[1]	Coconut oil	Olive oil	12C supplement	Oleostearine[2]	Oleostock	Safflower oil	18C supplement
8:0	8	0	4		0	0	0
10:0	7	0	4		0	0	0
12:0	51	0	30		0	0	0
14:0	19	0	11		2	0	3
15:0	0	0	0		1	0	0
16:0	8	12	10		25	8	26
17:0	0	0	0		2	0	2
18:0	1	1	1		22	1	25
14:1	0	0	0		1	0	1
16:1	0	2	1		4	0	4
17:1[3]	0	0	0		1	0	1
18:1	6	71	33		37	12	31
18:2	0	14	6	2	4	77	7
18:3	0	0	0	0	1	2	0
Saturated 8 to 14	85	0	49		2	0	3
Saturated 15 to 18	9	13	11		50	9	53
Total saturated	94	13	60	67	52	9	56
Total monoene	6	73	34	31	43	12	37
Total polyene	0	14	6	2	5	79	7

[1] The first figure represents the number of carbon atoms and the second the number of double bonds.

[2] Sample analyzed two years earlier by alkali isomerization.

[3] See footnote 2 to table 1.

and 2820 Cal., respectively. The fat extracted from each total diet was analyzed for fatty acids by gas-liquid chromatography and the data in terms of glycerides actually eaten are given in table 3. The fat from both diets contained about 2 gm of unsaponifiable lipid per man per day. The cholesterol content computed from tabular values was about 0.25 gm in diet 12C and 0.30 gm in diet 18C. The latter value includes 47 mg of cholesterol per 100 gm of 18-C supplement which is the equivalent of the Liebermann-Burchard reacting material of the digitonin precipitable sterols found by analysis. The lipid P of the extracted diet fats, purified by evaporating to dryness and redissolving in petroleum ether, was equivalent to about 2 gm of phospholipids per man per day.

Serum total cholesterol was determined by saponification of esterified cholesterol (Abell et al., '52), extraction of cholesterol into petroleum ether and development of color with a modified Liebermann-Burchard reagent (Anderson and Keys, '56). Phospholipids and the combined weight of total fatty acids and cholesterol were determined by methods described earlier (Keys et al., '60) except that the color development in the phosphorus determination was done by adding to the perchloric acid digest 3.3 ml of ammonium molybdate solution (8 gm per l) and 3.3 ml of dilute 1-amino-2-naphthol-4-sulfonic acid. The latter was prepared by diluting 20 ml of stock solution to 250 ml. The stock solution contained 30 gm of $NaHSO_3$, 6 gm of Na_2SO_3 and 0.5 gm of 1-amino-2-naphthol-4-sulfonic acid in 250 ml and was stored in the refrigerator. This method is that of Zilversmit ('58).

RESULTS

The serum cholesterol concentrations for all the men in both periods of experiment M are given in table 4. The apparent effect of diet change is given in the column headed "Δ Cholesterol 12C minus 18C, uncorrected." In group A most of the Δ values were negative and in group B most were positive. This inconsistent response reflects a time trend in serum cholesterol which was independent of the diets since both diets were used in each period. The mean cholesterol of all 27 men for the first period in which group A

TABLE 3

Fatty acids, as glycerides, in the two diets actually eaten in experiment AB (gm/man/day)

Fatty acids[1]	Diet 12C	Diet 18C	Excess	
			12C	18C
8:0	4	0	4	0
10:0	4	0	4	0
12:0	30	1	29	0
14:0	13	6	7	0
15:0	0	1	0	1
16:0	23	39	0	16
17:0	0	1	0	1
18:0	7	29	0	22
14:1	1	1	0	0
16:1	2	5		3
17:1[2]	0	1	0	1
18:1	51	46	5	0
18:2	8	9	0	1
18:3	1	1	0	0
Saturated 8 to 14	51	7	44	0
Saturated 15 to 18	30	70	0	40
Total saturated	81	77	4	0
Total monoene	54	53	1	0
Total polyene	9	10	0	1
Total glycerides	144	140	4	0

[1] The first figure represents the number of carbon atoms and the second the number of double bonds.

[2] See footnote 2 to table 1.

TABLE 4

Average serum cholesterol concentration for each subject in each period of experiment M and the differences (Δ cholesterol) caused by changing the diets (mg/100 ml)

Subject	Periods		Δ Cholesterol 12C minus 18C	
	1	2	Uncorrected	Corrected[1]
	Diet 18C	*Diet 12C*		
A1	190	176	−14	+5
A2	156	153	−3	+16
A3	212	188	−24	−5
A4	144	132	−12	+7
A5	170	156	−14	+5
A6	180	164	−16	+3
A7	246	203	−43	−24
A8	246	224	−22	−3
A9	189	184	−5	+14
A10	206	201	−5	+14
A11	156	171	+15	+34
A12	212	244	+32	+51
A13	208	215	+7	+26
A14	242	188	−54	−35
Mean for group A	197	186		
	Diet 12C	*Diet 18C*		
B1	222	186	+36	+17
B2	211	184	+27	+8
B3	230	200	+30	+11
B4	167	168	−1	−20
B5	192	144	+48	+29
B6	170	168	+2	−17
B7	248	204	+44	+25
B8	220	202	+18	−1
B9	178	154	+19	0
B10	208	188	+20	+1
B11	217	188	+29	+10
B12	173	156	+17	−2
B13	306	248	+58	+39
Mean for group B	211	184		
Mean for all men	204	185	+7.0	+7.7
S.E. of mean difference			±5.3	±3.5

[1] Corrected for time trend by increasing each value in period 2 by 19.

was receiving diet 18C and group B diet 12C was 204 mg per 100 ml. In the second period group A received diet 12C and group B diet 18C. The mean for all the men in this period was 185 mg per 100 ml. Except for the fact that group A had one more man than group B the situations regarding the experimental diets were strictly comparable. The difference between the mean cholesterol values of the two periods for the 27 men (204 − 185 = 19 mg per 100 ml) was apparently independent of the diets and has to be considered the consequence of some other factor which produced a mean decrease of 19 mg per 100 ml by the end of the second period. Such time trends are troublesome but by no means do they make it impossible to evaluate the effects of the diets themselves when a reversal design is used as in the present experiment. The statistical analysis of this type of experiment has been described by Brandt ('38) and is discussed in the text by Snedecor ('46). In this simple case the method of Brandt is almost exactly equivalent to adjusting the values by adding the difference between the means of the two periods (19 mg per 100 ml) to each value of the second period. As expected, this correction reduces the scatter of the individual differences, column 5 of table 4. The mean value of these differences, 7.7, closely approaches the mean value, 7.0, of the

uncorrected differences, the lack of identity reflecting the slight imbalance in the numbers of men (14 vs. 13) in the two groups. This illustrates how the reversal design detects the change due to experimental variables independently from extraneous uncontrolled factors. The effect of the correction is to decrease the variance of the values by eliminating almost all the variance due to a nondietary time trend. This reduces the standard error of the mean difference as shown by the values given in the table.

Using these differences based on the corrected serum cholesterol values a statistical test was made to find whether the mean difference was significantly different from zero. The value of *t*, obtained by dividing the mean difference by its standard error was 2.2 and the probability of chance occurrence of a mean difference as large as that found was $P = 0.04$. The only departure of this method of calculation from that of Brandt consists in the use of 26 degrees of freedom in place of 25 and in the rounding of the time trend correction. The data show then that the men had a higher serum cholesterol while eating diet 12C but the experiment was repeated in order to achieve greater security.

A summary of the serum lipid observations of experiment AB is given in table 5. Serum cholesterol data were available for all 23 men. The data indicate that diet 12C produced a serum cholesterol level higher by 9.0 mg per 100 ml than diet 18C and that this difference was significant at $P = 0.005$. The mean changes in serum cholesterol level between periods independent of the dietary treatments were only 7 mg per 100 ml. If correction for this time trend is applied as done with the data of the previous experiment the value of P remains the same.

In experiment AB, analyses were made of the serum of 16 men to determine phospholipid, and the total fatty acids in addition to total cholesterol. From these data triglyceride and total lipid concentrations were calculated. These 16 men were selected at the beginning of the experiment on the basis of their serum cholesterol concentrations. After all 23 men had been divided into the 4 matched experimental groups, the two individuals with the highest serum cholesterol levels of each group and two with the lowest were selected for these determinations. The findings with these men and the cholesterol values of the same 16 men, are summarized in table 5.

The difference in mean serum phospholipid level was 9.3, almost identical to that for cholesterol, but the variability of the data was so great that the difference cannot be considered significant. The differences in serum triglycerides and in serum total lipid must also be considered nonsignificant. When only the 15 men with the

TABLE 5

Serum lipid values (means and standard deviations) for all 23 men or for 16 selected men, in experiment AB

	No. of men		Diet 12C	Diet 18C	Δ[1]	S.E.[2]	P[3]
Total cholesterol	23	Mean	196.4	187.4	+9.0	±3.0	0.005
		S.D.	27.7	27.9			
Total cholesterol	16	Mean	194.1	184.9	+9.2	±4.1	0.04
		S.D.	30.3	28.9			
Phospholipid	16	Mean	218.7	209.4	+9.3	±6.3	> 0.1
		S.D.	34.4	34.8			
Triglyceride	16	Mean	101.0	125.2	−24.2	±12.6	0.08
		S.D.	43.9	66.8			
Total lipid	16	Mean	607.2	609.2	−2.4	±19.9	> 0.1
		S.D.	99.6	114.7			

[1] Δ indicates the mean difference due to exchanging diets.
[2] S.E. is the standard error of the mean difference.
[3] P is the probability of chance occurrence of a mean difference as large as Δ.

highest initial serum cholesterol were considered, omitting the lowest 8, the mean difference in serum cholesterol level, 12C minus 18C, was 10.5 mg per 100 ml and the probability of chance occurrence 0.003. This greater difference with improved statistical significance is to be expected from the generalization that subjects with high intrinsic serum cholesterol levels respond to dietary alterations with large changes in serum level (Keys et al., '59).

DISCUSSION

It will have been noted that in both experiments M and AB the matching between diets 12C and 18C though very close, was not exact. In experiment M, diet 12C contained an average daily excess of 4 gm of saturated, whereas diet 18C provided an excess of 1 gm of polyene glycerides.

The expected effects of these trivial differences can be estimated with the prediction equation mentioned at the outset derived by the method of least squares from many other controlled experiments:

$$\Delta \text{ Cholesterol} = 2.68\ \Delta\ S - 1.23\ \Delta\ P$$

in which Δ S is the difference between two diets in percentage of calories provided by saturated glycerides, Δ P is the difference provided by polyene glycerides and Δ cholesterol the difference in cholesterol, mg per 100 ml of serum, on the two diets. Application of this equation to the present dietary data gives expected values for experiments M and AB, respectively, of +1 and +2 mg of cholesterol per 100 ml for diet 12C minus diet 18C. Correcting for these predictions slightly reduces the statistical significance of the differences observed but the conclusions are not altered.

The essential identity of the two experiments with regard to subjects, diets and results means that the two sets of data can be pooled for analysis of 50 comparisons between cholesterol values for diets 12C and 18C. The result is an average cholesterol value 8.3 mg per 100 ml higher with diet 12C than with diet 18C, with $P = 0.001$. This pooling was made using the corrected data of experiment M and the uncorrected data of experiment AB. When the data of experiment AB were corrected for time trend and pooled with the corrected data of experiment M the mean and the probability of chance occurrence were practically the same as before. If the results are corrected for the slight mismatch in the diets, the difference is 6.8 with a value of $P < 0.001$.

Hashim and others ('60) have recently reported that the addition to the diet of a fat mixture of glycerides of "medium" chain length saturated fatty acids, mainly 8-carbon, produces lower serum cholesterol levels than isocaloric amounts of butterfat. In the experiments reported here such "medium" chain length fatty acids represented a maximum of only 8% of the total fatty acids in the diet (diet 12C). It is interesting that the cholesterol differences obtained by Hashim and his co-workers, when comparing corn oil and butterfat diets, agreed with those predicted from the above mentioned equation. The observed difference in the average serum cholesterol level when corn oil and butterfat were exchanged was 68 mg per 100 ml; the predicted value would be 64.

Experiments, both on animals (Bloom et al., '51; Kiyasu et al., '52) and man (Fernandes et al., '55) indicate that fatty acids with fewer than 12 carbons in the chain are transported from the intestine to the blood via the portal vein, whereas fatty acids of longer chain length are transported via the lymphatics. These differing mechanisms may help to explain the difference in the effect on the blood cholesterol level of the fatty acids of different chain length.

Considerations of degree of saturation and of chain length of the fatty acids do not easily explain the result reported by Ahrens and others ('57) which indicated lower serum cholesterol values with a cacao butter diet than when that fat was isocalorically replaced by butterfat. These two fats have almost identical iodine values and composition in terms of fatty acids classed in groups as saturated, monoene and polyene. The most obvious difference between these two fats is that about one third of the saturated fatty acids in butterfat have 14 or fewer carbon atoms in the chain, whereas in cacao butter almost all of the saturated fatty acids are C16 and

C18 in length. Otherwise most of the experimental findings so far available on man would be consistent with the theory that among the saturated fatty acids the serum cholesterol-raising effect decreases moderately with increasing chain length beyond C12 (lauric) and sharply decreases with decreasing chain length below C12 to essentially zero effect with butyric acid.

Following a method of computation similar to that used in the derivation of the prediction equations it is possible to make an approximate evaluation of the effect on serum cholesterol of the 12- and 14-carbon saturated fatty acids provided a value can be given to the effect of the 8- and 10-carbon saturated fatty acids. From the data of Hashim it is apparent that the effect of the 8- to 10-carbon saturated fatty acids on serum cholesterol lies in the range of the monoene or polyene fatty acids. The coefficient expressing its activity should then have a value between zero and -1.2. Under this assumption the value of the coefficient for the 12- and 14-carbon saturated fatty acids must be between 3.7 and 4.0. This value is clearly higher than the value 2.68 which has been found to apply to the 16- and 18-carbon saturated fatty acids which predominate in most food fats. It has been observed that the saturated fatty acid mixture of butterfat has an effect on serum cholesterol concentration corresponding to the coefficient 2.68. When the preceding data are applied to the composition of butterfat it is easy to see that the high cholesterol-promoting effect of the 12- to 14-carbon saturated fatty acid is counteracted by the behavior of the shorter chain saturated fatty acids.

SUMMARY

The influence of the carbon chain length of the saturated fatty acids of dietary fats on their effect on serum cholesterol concentration was studied in two controlled experiments. In each experiment two groups of physically healthy middle aged men ate two diets containing approximately 3000 Cal. and 140 gm of total fat. The fats of the two diets were similar as to the proportion of saturated, monoene, and polyene fatty acids but one of the diets (12C) had about 40 gm more of lauric and myristic acids than the other, whereas the second diet (18C) had about 40 gm more of palmitic and stearic acids than the first. The diets were fed alternately for periods of three weeks to all the men and blood samples taken on two days at the end of each dietary period. The results of 50 comparisons between the diets showed that diet 12C, containing a higher proportion of saturated fatty acids of 12 and 14 carbon atoms, produced a serum cholesterol concentration higher by 8 mg per 100 ml than diet 18C.

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Effects of Long-Term Feeding of Fat-Free Diets to *Cebus* Monkeys[1]

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Since the original observations by Burr and Burr ('29) and by McAmis et al ('29) that rats fed diets devoid of fat grew poorly and developed deficiency signs, and the demonstration that these defects could be reversed by the inclusion of fat or certain fatty acids (Burr and Burr, '30) in the diet, "essential fatty acid" deficiency has been produced in a wide variety of species. These include certain insects, chickens, mice, hogs, calves, dogs, and human infants (cf. review of Deuel and Reiser, '55). It has been the experience of various workers that the deficiency is most easily produced in young growing animals and in adult animals in which the body stores of essential fatty acids (EFA) are relatively low. The single experiment by Brown et al. ('38) in which a male adult human was subjected to a fat-free diet for 6 months failed to demonstrate any signs of deficiency. In fact, the studies of Hirsch et al. ('60) indicate that the linoleic acid concentrations of adipose tissue of man change very slowly when a fat-free diet is introduced. There is, on the other hand, no direct evidence that significant quantities of linoleic acid or other fatty acids of the linoleic or linolenic acid series are synthesized by any mammalian tissues. Theoretically, all tissues can eventually be depleted of EFA when a diet devoid of these acids is fed.

More recently, because of the widely recognized effect of highly unsaturated fats, including those rich in EFA, on the serum cholesterol level (review of Ahrens, '57), there has been an interest in the metabolic interrelationships of cholesterol and EFA. The feeding of cholesterol accelerates the induction of EFA deficiency signs in rats (Holman and Peifer, '60). It has also been observed (Alfin-Slater et al., '54) that rats fed diets devoid of fat have significantly lower serum cholesterol levels but higher concentrations of liver and adrenal cholesterol esters than controls fed diets containing fat. There is no evidence for or against such an inverse relationship between serum and other tissue cholesterol concentrations in man fed diets low in fat or devoid of fat.

This study involved a comparison of *Cebus* monkeys fed diets devoid of fat and those fed diets containing corn oil for periods of up to two years. The fatty acid composition of certain tissue lipids of *Cebus* monkeys has already been shown to reflect rapidly the institution of a diet low in EFA (Portman et al., '59a, '59b). This rapid response is probably due in part to the remarkable leanness (small adipose tissue pool size) of the *Cebus* monkey. Until the present study there were no available data on the adipose tissue of *Cebus* monkeys, since the animals have no appreciable subcutaneous adipose tissue or other site of easy biopsy of adipose tissue.

There were two aspects of this study: (1) an evaluation of the clinical, biochemical, and histological evidence of a requirement for EFA by *Cebus* monkeys; and (2) the determination of the long-term effect of feeding diets devoid of fat on the free and ester cholesterol concentration in a wide variety of tissues.

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EXPERIMENTAL

Thirteen *Cebus* monkeys were used in this study. Most of them were of a size at the beginning of the experimental period, which would, on the basis of previous experiments, indicate early sexual maturation, and all were gradually increasing in body weight. Nine of these monkeys were fed diets devoid of fat and 4 were fed a diet containing 8% by weight (14% of calories) as corn oil (control diet). The control diet was the basal purified diet used in this laboratory over a period of about 12 years (Mann et al., '53) and has been fed for various periods to approximately 300 monkeys. The fat-free diet was prepared so that the ratios of protein and vitamins to calories were identical to the control diet. Supplementary vitamin E was not added to either diet. Thus the monkeys receiving the corn oil diets received substantial quantities of tocopherols, whereas the monkeys receiving the fat-free diets did not. The decision not to make the two diets equivalent in tocopherol content was made on the basis of the marked diminution in tocopherol requirements by animals fed fat-free diets. The use of equivalent quantities of vitamin E in the two diets would, perhaps, not be justified. Nevertheless, the very low concentrations of vitamin E in the fat-deficient diet must be kept in mind in the interpretation of this study.

The monkeys were weighed weekly in the morning before receiving the daily meal and were usually bled once monthly for determinations of serum cholesterol and other lipid values.

Five monkeys (one male, two females, and two male castrates) from the essential fatty acid-deficient group were sacrificed after receiving the experimental diet for 7, 15, 18, 19, and 22 months, and 4 monkeys (one male, two females, and one male castrate) from the control group were sacrificed after being supplied with the corn oil-containing diet for 8, 16, 18, and 25 months. Intravenous sodium amytal was administered and the monkeys were sacrificed by opening the thorax and drawing a large volume of blood from the right side of the heart. Individual organs were weighed and aliquots for chemical analysis and histological examination were taken as rapidly as possible. The remaining 4 monkeys fed the fat-free diet were sacrificed for limited histological evaluations only.

The tissue aliquots for chemical analysis were homogenized in chloroform: methanol (2/1; v/v), incubated at 40° for one hour, brought to volume, and filtered into culture tubes with Teflon-lined caps. Samples were stored at − 20° or at 5° in nitrogen filled dessicators until analyses were performed. The isolation of β-lipoproteins from serum by ultracentrifugal techniques (Gofman et al., '49) was begun on the same day that the serum was obtained. An aliquot of the β-lipoprotein preparation was treated with the chloroform-methanol solution within 18 hours of obtaining the serum.

The lipids from some of these tissues were further separated into three fractions by a silicic acid column chromatographic procedure which has been previously described (Portman et al., '59a). Cholesterol determinations (Abell et al., '52) were performed on the cholesterol ester and free cholesterol fractions. Fatty acids obtained from the saponifiable components of the silicic acid column fractions or from the total lipid fractions were methylated and separated by gas-liquid chromatography, using a 4-foot column with polyethylene glycol adipate or succinate stationary phase and an argon ionization detector.[3] The fatty acids from nervous tissue lipids were also analyzed for the various classes of polyunsaturation by the microalkali isomerization technique of Holman and Hayes ('58). This latter procedure was used to supplement the gas-liquid chromatographic procedure. Total fatty acids in various fractions were determined by the titrimetric procedure of Entenman ('57).

For histologic studies tissues were fixed in 10% neutral formalin. Hearts and aortas were stained grossly with Sudan IV and examined for lipid-bearing lesions by a modification of the method described previously (Mann et al., '53). Complete microscopic studies were carried out including all endocrines, the central nervous system, peripheral nerves, eyes, and others.

[3] Obtained from the Pye Instrument Company, Cambridge, England.

Alcian blue, hematoxylin, and eosin were combined as a general staining technique. Pituitary sections were stained with PAS-orange G and also with aldehyde fuchsin-trichrome, following formol sublimate mordanting. Frozen sections of skin, liver, kidney, adrenal, heart, and aorta were stained with Sudan IV-hematoxylin.

RESULTS

Weight gain and clinical picture. There were relatively few objective signs of a deficiency syndrome in the *Cebus* monkeys fed a fat-free diet. The fat-free monkeys remained active and alert throughout the experimental period. Within 4 to 6 months of the institution of the fat-free diet, changes in the hair and skin became evident. There was thinning of the hair on the tail and in some cases over the frontal area of the head. There was minimal hair thinning over the anterior thorax and thighs. The color of the hair on the ventral surface, which in this species was normally light tan, changed to a yellow to orange and in one case to a reddish hue. The skin of the tail became dry and tended to desquamate noticeably. The tail skin of several monkeys became encrusted and in one of these monkeys there were small superficial ulcerations on the tail and over the palms and soles.

The weight curves of one of the lightest and of one of the heaviest monkeys from the fat-free group and of similar examples from the controls fed corn oil are illustrated in figure 1. One of the monkeys included (no. 128) had received a fat-free diet for 6 months prior to institution of the control diet and a very sharp increase in

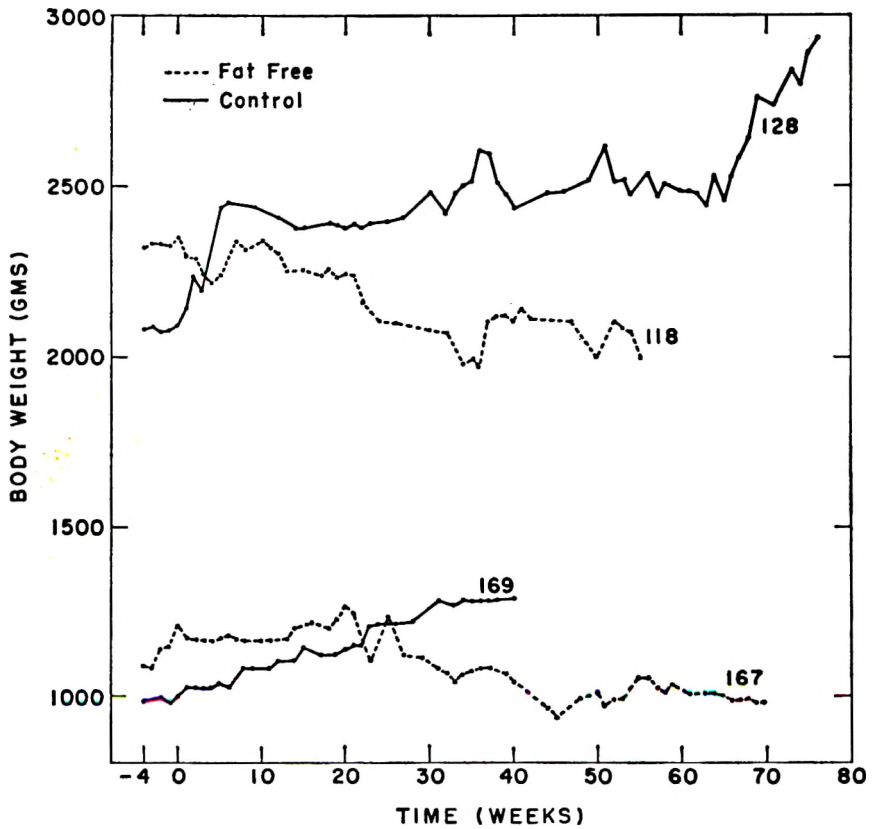

Fig. 1 The effect of feeding fat-free diets and diets containing corn oil on the body weight of *Cebus* monkeys. One of the heaviest and one of the lightest monkeys from each group are illustrated. The indicated diets were instituted at time zero. Monkey no. 128 had been fed a fat-free diet until time zero when the corn oil diet was instituted.

weight was observed immediately after the diet change. There was a slow accumulation of weight in the control group and a small loss of weight in the fat-free group. At three months the mean weight gain of the 9 monkeys in the fat-free group was + 23 gm and at 6 months, − 31 gm. For the 4 monkeys of the control group the figures were + 208 gm and + 241 gm at three months and 6 months, respectively. Because of the difficulty in accurately measuring food and water intake of monkeys, it was impossible to demonstrate polyphagia and polydipsia (characteristic of fat-deficient rats [Burr and Burr, '30]) in the fat-deficient group. There was a distinct impression that the fat-deficient monkeys consumed more food on a caloric basis than did controls fed corn oil.

Fatty acid composition of various tissue lipids and lipid subfractions. Usually 24 different tissues from each monkey were taken for determination of the distribution of fatty acids in the lipid fractions. In some instances these lipids were further fractionated into three major classes before the methyl esters of the constituent fatty acids were prepared and chromatographed in the gas-liquid system.

The fatty acid analyses of many of the various tissues from the same monkey were quite similar. This is undoubtedly due to the fact that the adipose tissue in the omental and mesenteric fat and the interstitial triglyceride which is prominent in certain of the parenchymal organs evaluated were metabolically identical. On the other hand, certain of the parenchymal tissues and, of course, nervous tissue had unique fatty acid distributions, presumably a reflection of their high concentrations of other complex lipids. In table 1 are shown the fatty acid distributions in certain tissues from one monkey fed a fat-free diet for 19 months and one monkey fed an otherwise identical corn oil-containing diet for 16 months. These values are qualitatively representative of those obtained in analyses of tissues from the other monkeys studied.

The sera and liver lipids have been further fractionated into cholesterol esters, glyceride-free fatty acids, and phospholipids prior to fatty acid analysis. The decrease in linoleic[4] acid concentrations and the increase in palmitoleic and oleic acid concentrations in the lipids from the fat-deficient monkey (compared with corn oil controls) are readily apparent. Significant although reduced concentrations of a fatty acid with retention time consistent with that of linoleic acid were still present in heart, intestinal mucosa, red blood cells, ovaries, and leg skin even after the animals had received the fat-free diet for 19 months. Linoleic acid was present in very minimal quantities in liver, serum, and adipose tissue. The phospholipid fraction of liver, however, still contained about 4.5% of arachidonic acid. There was an accumulation of a 20-carbon fatty acid of retention time less than that of arachidonic acid (presumably 5, 8, 11- or 7, 10, 13-eicosatrienoic acid, Mead, '60) in tissues of the fat-deficient monkeys. This acid represented 17.5% of the fatty acids of liver phospholipid from the fat-deficient monkey of table 1.

Polyunsaturated fatty acids of brain lipids. There was no consistent difference in the concentrations of fatty acids of chain length shorter than 20 in the lipids of brain from the two groups of monkeys. We had not identified with sufficient certainty the longer chain fatty acids of brain by gas-liquid chromatography. The alkali isomerization procedure was, therefore, used to identify any differences between the polyunsaturated fatty acid compositions of brain lipids of the two groups of monkeys. In table 2 are shown the differences in polyunsaturated fatty acid composition of lipids from various samples of nervous tissue from 4 monkeys fed diets containing corn oil and from 4 monkeys fed fat-free diets for 7 to 22 months. Values are expressed as the ratio of trienoic to tetraenoic acids. There was a decidedly greater triene/tetraene ratio for the fat-deficient monkeys than for any of the control monkeys fed corn oil. This ratio was increased in the nervous tissues of the monkey fed a fat-free diet for 7 months, was around 0.9 in the monkey fed a fat-free diet for 15 months, and was greater than 1.0

[4] There is no proof of the identity of these acids beyond the fact that their relative retention times are consistent with those obtained using pure samples and with the published values of others (e.g., Farquhar et al., '59).

TABLE 1

Distribution of fatty acids in the total lipid or lipid sub-fractionsfrom tissues of a Cebus monkey fed a fat-free diet for 19 months and the control (8% corn oil) diet for 16 months

Fatty acid[1]	Liver[2]			Serum			Serum β-lipoprotein	Heart	Kidney	Intestinal mucosa	Mesenteric fat	Leg skin	Adrenal	Pancreas	Ovaries	Intima aorta	Red blood cells
	CE	TG	PL	CE	TG	PL											
Fat-free diet for 19 months																	
12:0								0.8	0.8		tr.	1.9	2.0		tr.	2.6	0.9
14:0	2.0	1.7	0.4				5.0	2.4			3.2	1.8	tr.	3.7		2.8	3.9
14:1				1.6							0.6						
15:0	1.4	0.7		1.1		1.2			2.8	2.9	0.5		tr.		1.8		7.6
16:0	20.1	26.8	21.6	18.4	21.8	26.0	17.2	13.2	19.5	17.7	24.5	12.3	16.2	44.6	26.3	17.8	14.5
16:1	12.3	8.4	7.5	10.8	6.6	7.6	1.0	2.2	4.1	2.1	8.5	3.7	5.4	15.2	9.4	3.3	14.7
17:0				1.2				8.4		10.0	1.1	8.0				3.7	
18:0	7.0	4.5	16.8	1.4	11.0	10.8	9.6	20.0	15.4	18.8	11.8	13.0	9.6	20.9	14.0	54.0	16.7
18:1	57.2	53.0	31.2	65.5	60.6	32.4	50.6	15.0	33.7	15.6	47.3	23.7	43.9	10.4	39.8	16.8	13.4
18:2						1.1	tr.	11.4	2.0	13.1	1.3	13.2	tr.		8.8	tr.	
20:0								10.0	tr.	9.0	1.1	13.3				tr.	11.2
20:1		2.1					1.2				tr.		3.2				
20:2																	
20:3		3.2	17.5			19.7	9.3	14.0	20.6	10.8	tr.	9.1	9.3	5.1			12.5
20:4			4.5				0.6				tr.		tr.				4.7
Control (8% corn oil) diet for 16 months																	
12:0			0.1			tr.	tr.	tr.	tr.	tr.	tr.	1.0	tr.			2.2	
14:0	tr.	1.8	0.7	tr.				tr.		0.6	1.8	2.1	0.7	0.7	2.6	11.0	0.9
14:1		tr.		tr.	tr.	tr.						tr.					1.7
15:0	2.6	0.9	1.7	tr.	tr.	tr.		5.5	1.9	2.2	0.3	3.1	tr.	0.2		5.5	1.1
16:0	20.6	20.0	17.8	10.7	18.3	26.6	17.0	15.0	22.9	15.2	13.4	5.8	13.2	12.2	26.8	25.9	21.7
16:1	5.6	4.5	1.9	4.9	4.6	6.3			tr.	17.1	2.0	9.4	3.2	1.7	2.6	5.8	1.9
17:0		tr.	0.5	tr.		tr.		1.7			0.6	9.5	tr.	0.4			1.7
18:0	7.0	7.4	18.9	7.7	7.2	22.2	7.5	29.8	20.1	19.0	11.8	4.6	7.2	9.1	19.9	8.5	30.4
18:1	28.1	24.2	7.3	43.6	26.3	15.2	24.2	12.0	15.5	8.4	27.1	20.4	38.0	33.2	27.4	28.6	8.0
18:2	36.3	41.2	24.2	33.1	43.7	29.6	51.3	36.0	22.8	29.4	40.1	10.3	32.4	40.6	10.5	12.4	7.2
18:3											0.5	11.2?					7.6?
20:0								10.0		7.5	0.5	10.8				tr.	tr.
20:1			3.8								0.8						6.4
20:2											0.8	8.1	4.4				tr.
20:3			2.7							7.0	0.7		4.9	0.9			
20:4			14.1					14.0	16.8	3.6		3.6	3.2	1.1	10.1		11.2

[1] The designation for fatty acids is chain length : number of double bonds.

[2] Fatty acids from cholesterol ester fraction (CE), triglyceride (TG), and phospholipid (PL).

TABLE 2

Ratio of trienoic to tetraenoic acids in the total lipid extract of nervous tissue from fat-deficient and control Cebus monkeys

	Fat-deficient[1]	Control[1]
Cerebrum	1.02 (0.66–1.36)[2]	0.34 (0.17–0.42)
Cerebellum	0.91 (0.45–1.15)	0.43 (0.27–0.57)
Medulla	1.00 (0.70–1.46)	0.47 (0.27–0.65)
Pons	1.23 (0.73–1.87)	0.43 (0.37–0.49)
Peripheral nerve	1.60 (1.02–2.96)	0.53 (0.28–0.77)

[1] N=4 monkeys per group.
[2] Mean value (range of values).

TABLE 3

Fatty acid composition of total phospholipid fractions from 6 tissues of a monkey fed fat-free diets for 15 months

	Liver	Kidney	Adrenal	β-Lipoprotein serum	Whole serum	Red blood cells
14:0	0.31	1.24	0.22	1.56	1.50	0.56
15:0	tr.	3.13	0.37	1.04	1.88	tr.
			0.98			1.48
						0.41
16:0	20.4	15.1	16.2	30.8	24.6	17.4
16:1	4.99	2.22	1.67	9.36	7.29	4.37
17:0	tr.	2.02	1.82	tr.	tr.	tr.
					tr.	tr.
18:0	16.5	15.0	14.7	12.2	9.81	20.0
18:1	31.6	29.4	19.8	22.7	32.2	19.6
18:2	1.19	3.29	0.66			
20:1			0.70			
20:2			0.55			
20:3	20.6	18.2	14.9	16.4	22.6	19.6
20:4	4.28	10.28	14.7			17.5
Unknown (22:1)			4.01			
Unknown (22:4)			2.60			
Unknown (24:0)			2.06			
Unknown (22:5)			1.22			
Unknown (24:1)			2.67			

(compared with mean values of around 0.4 for control monkeys) in two longer term monkeys. Examination of the nervous tissue lipids from the longer term fat-deficient monkeys by gas-liquid chromatography indicated that around 5% of the total fatty acids were eicosatrienoic (20:3) with only traces of an eicosatetraenoic acid. Control tissues (from monkeys fed corn oil) indicated an exact reversal of this pattern.

Heterogeneity of composition of total phospholipid fatty acids in different tissues of the same monkey. As the data in table 1 indicate, there was a wide range of fatty acid compositions in the total lipid extracts of different tissues from the same monkeys even after these animals had been fed the same diet for long periods. This is in general agreement with the results in a variety of studies in other species.

The phospholipid components of various tissues are usually considered to be important in "structural" roles and are thought to be more resistant to change in fatty acid composition (Thompson and Ballou, '56). Data in table 3 indicate the distribution of fatty acids in the total phospholipid fractions from 6 tissues of a monkey fed a diet devoid of fat for 15 months. There was somewhat greater similarity in the analyses of phospholipids from different tissues than there was in the analyses of total fatty acids from those tissues. There were still traces of linoleic acid measurable in the liver, kidney, and adrenal but not in the red blood cells, serum, or serum

TABLE 4

Mean concentrations of free and ester cholesterol in a variety of tissues from fat-deficient and control Cebus monkeys (mg/gm of wet tissue)

	Fat-deficient[1]		Control[2]	
	Free	Ester	Free	Ester
Serum	0.32	0.90	0.45	1.57
β-Lipoprotein	0.17	0.46	0.18	0.72
Liver	2.30	0.80	2.67	1.05
Kidney	6.58	0.58	5.82	0.45
Adrenal	4.59	19.94	5.77	37.11
Spleen	3.82	0.59	4.45	0.93
Ovary	2.56	4.95(2)[3]	10.24	2.80(2)
Testis	2.41	2.62(1)	2.04	0.88(1)
Muscle	0.74	0.09	0.86	0.02
Chest skin	2.51	2.01(3)	1.25	0.38(3)
Leg skin	4.21	2.38(3)	2.62	1.69(2)
Aorta-intima	2.45	1.28	1.68	0.48
Intestinal mucosa	2.77	0.70	3.58	0.33
Thymus	1.34	0.40(2)	3.70	0.48
Pancreas	2.50	1.26	3.95	1.23
Lung	4.09	0.65(2)	2.78	0.42
Medulla	31.02	0.00	46.22	0.02
Pons	36.30	0.04	33.53	0.02
Cerebellum	15.09	0.05	20.39	0.02
Cerebrum	18.59	0.03	21.58	0.00
Nerve	16.09	0.77	28.85	0.15
Heart	1.24	0.04	1.47	0.08

[1] N = 5 monkeys per group.
[2] N = 4 monkeys per group.
[3] Where analyses were not available on all monkeys, numbers in parentheses are the numbers of analyses performed.

β-lipoprotein phospholipid. Substantial amounts of arachidonic acid were present in the phospholipids of liver, kidney, adrenal, and red blood cells. A major peak in all 6 phospholipid fractions was a methyl ester of an acid with a relative retention time (about 2.65 at 180°) which was, probably, 5, 8, 11- or 7, 10, 13-eicosatrienoic acid. A number of unidentified fatty acids of relative retention times greater than that of arachidonic acids were present in the adrenal phospholipid fraction.

Concentrations of free and ester cholesterol in tissues. In table 4 are shown the mean concentrations of free and ester cholesterol in various tissues from 5 monkeys fed fat-free diets and 4 monkeys fed corn oil-containing diets. Individual values are not indicated for either group, since there was no indication of a difference in concentrations related to duration of the feeding periods. Because of the variation in values for a given tissue in each group, it was usually impossible to establish high statistical significance of small differences between the two groups of monkeys. The concentrations of total and ester cholesterol were less in the serum of the fat-deficient group throughout the feeding period and at the time of sacrificing. This finding is consistent with prior observations on EFA deficiency in rats. In contrast with EFA deficiency in rats, however, fat deficiency in monkeys did not result in elevated terminal cholesterol ester concentrations in liver or adrenals. The cholesterol ester concentration in the adrenals of the series of fat-deficient monkeys was lower than the mean value for control monkeys. The concentrations of free and ester cholesterol in aorta, leg skin, and chest skin were higher in the fat-deficient group than in the control group. Because of the complex pattern of skin sterols and the non-specificity of the cholesterol method employed in these studies, the values for skin cholesterol must be considered with reservation. The same limitations also apply to the cholesterol values for certain of the other tissues. The mean cholesterol concentrations in most of the other tissues were similar for the two groups.

TABLE 5

Organ weights (expressed as percentages of total body or of kidney weight)

	Fat-deficient[1]	Control[1]
Liver/body	3.27	2.21
Brain/body	4.57	3.34
Heart/body	5.51	5.67
Kidneys/body	0.69	0.54
Adrenals/kidneys	4.8	4.0
Thyroid/kidneys	1.9	2.0
Gonads/kidneys	3.8(O)[2]	5.3(O)
	25.9(T)	61.3(T)

[1] N = 4 monkeys per group.
[2] O indicates ovaries; T, testes.

Total fatty acid concentrations in tissues. Total fatty acid concentrations were also measured in the tissues indicated previously under cholesterol concentrations. This measurement could be expected to serve as a comparative index of total lipid concentration. There was invariably a slightly lower mean concentration of total fatty acids in the tissues of the fat-deficient group. Because of the small size of the series involved the differences were of a low order of significance.

Morphological considerations — organ weights (absolute weights and proportions of body weight). In table 5 are shown the mean values of certain organ weights for the monkeys fed the fat-free and those fed corn oil-containing diets. Because of the wide disparity of total body weights within and between groups, the weights are expressed as a proportion of total body weight or as a ratio to kidney weight. The liver, kidneys, and total brain (expressed as a percentage of body weight) were larger in the fat-free group. When these organ weights were expressed as a percentage of the pre-experimental body weights, there was no significant difference in the mean values of the two groups of monkeys. It is quite probable then that the values shown in table 5 could properly be interpreted as resulting from changes in body weight during the experimental period rather than as a result of organ hypertrophy. The mean heart weights of both groups as a percentage of body weight were not different. Other organ weights considered as ratios to kidney weights were similar for the two groups of monkeys, except that the fat-deficient monkeys had lighter gonads (two females, one male per group).

Pathologic findings. No gross changes pertinent to this experiment were seen at autopsy other than the skin changes noted above. There was an apparent decrease in body adipose tissue in the fat-deficient monkeys though this was difficult to evaluate because of the very scant amounts of such fat present in control monkeys and that regularly observed in other *Cebus* monkeys.

Significant histologic changes were limited to the skin, bone marrow, reticuloendothelial system, and gonads. Of the skin samples (three areas sampled) those from 6 fat-deficient monkeys showed definite hyperplasia of the epidermis, two showed questionably significant changes, and one was indistinguishable from control specimens. This hyperplasia consisted essentially of acanthosis with an increase in the number of cells in the germinal layer, but also with some increase in the granular layer. The number of cells was doubled or tripled as compared with control skins. In marked examples rete peg formation was striking and the process extended into the hair follicles but without involvement of the adnexal glands. The germinal layer showed an increased prominence of the intercellular spaces and a slight increase in numbers of mitoses. There was no parakeratosis or hyperkeratosis, the keratohyalin layer, if anything, being somewhat less conspicuous than that seen in control tissues. There were no consistent changes in the thickness of the corium or its fat content and no specific changes were seen in the ground substance or collagen. Focal ulceration of the epidermis accompanied by extensive acute inflammation was seen in one EFA-deficient monkey.

Skin changes, when present, were generally seen at all three sites sampled, namely, thorax, abdomen, and calf. The relative degree of involvement at these sites was quite variable among the different animals. In comparing prior biopsy with autopsy specimens progression of the lesion with time was apparent. The one experimental monkey showing no detectable skin changes however, had been fed the deficient diet longer than any other, namely, 22 months.

Bone marrow from the upper end of the femur was available from 4 of the 5 sacrificed EFA-deficient monkeys and from three of the 4 controls. All 4 of the former specimens showed a distinct hyperplasia with replacement of the usual fat deposits by highly cellular marrow. In three instances the process appeared restricted to the erythropoetic series, with increases in numbers of cells of varied developmental stages, particularly late nucleated forms. In the 4th specimen there appeared to be an increase in segmented forms of the myelopoetic series as well. Extramedullary erythropoesis was seen in the livers of two of the 5 fat-deficient monkeys. In one of the fat-deficient monkeys this process was quite marked. This animal showed splenic erythropoesis, the spleen in this instance being the largest of all the animals and weighing 3.61 gm with a spleen-kidney weight ratio of 0.25.

Erythrocytophagocytosis as seen throughout the reticuloendothelial system, but most readily visualized in livers and lymph nodes, was seen in both groups of animals though somewhat more prominently in the fat-deficient groups. Hemosiderin deposition was not correlated with either the degree of erythrocytophagocytosis, the presence of marrow hyperplasia, or the dietary grouping.

Of the 5 deficient monkeys which were subjected to detailed histological examination, two were female, one was male, and two were male castrates. The three intact animals should have been sexually mature by comparison of organ weights with data from *Cebus* collected in the wild.[5] Of the two females one showed a large hemorrhagic corpus luteum. The ovaries of the other animal weighed 0.34 gm and although being in the size range of sexually mature females (0.25 to 1.70 gm) contained no recognizable corpora lutea. The testes of the one intact male weighed 3.8 gm as compared with 8.8 gm for the one control male and the range of 4.6 to 8.3 gm from sexually mature wild specimens, all of the same general developmental age as the deficient male. Histologically the seminiferous tubules of this animal showed only Sertoli cells, whereas the interstitial cells were small, of increased numbers, more heavily pigmented, and less eosinophilic than those of active testes. This animal showed several other isolated findings not seen in the remaining animals. There was a distinct loss of adrenal cortical lipid, especially in the zona reticularis. Squamous metaplasia was present in the renal pelvis, the prostatic urethra, and the cornea, accompanied, in the latter two sites, by acute inflammation.

In the remaining animals no constant changes relevant to fat deficiency could be seen in the appearance of the adrenal cortices or in the zonal distribution of lipids. The pituitary and thyroid glands showed no changes pertinent to this experiment. No significant deviations from the control animals were seen in the aortas or other arteries, kidneys, or gastrointestinal tract (sections from 4 to 5 levels). There was no increased lipid accumulation in livers of the fat-deficient monkeys. Changes in the remaining tissues were incidental and presumably unrelated to the experimental regimen.

DISCUSSION

A requirement for dietary fat, and specifically a requirement for fatty acids of the linoleic acid series (Mead, '60), has been demonstrated in almost all species which have been fed fat-free or EFA-deficient diets for a sufficiently long period. There is, however, relatively little information concerning a requirement for EFA by primates except by human infants.

The studies presented here indicate that *Cebus* monkeys show certain clinical, histological, and chemical changes when a fat-free diet is fed. The principal clinical and histological changes were seen in the skin and hair. The nature and distribution of these changes is consistent

[5] Andrus, S. B., unpublished data.

with those seen in rats by Ramalingaswami and Sinclair ('53) and other workers, although hyperkeratosis and changes of the sebaceous glands were not noted. Only one male, which on the basis of size and organ weights should clearly have been sexually mature, was in the fat-deficient group. This animal demonstrated aspermia and abnormally small testes. This observation is consistent with that of Aaes-Jorgensen et al. ('56) in deficient rats. The possibility that the testicular atrophy was due to vitamin E deficiency must also be considered. Ovarian and pituitary changes as described by Panos and Finerty ('53) in fat-deficient rats and interpreted as reflecting decreased secretion of estrogen and luteinizing hormone were not seen. One of the fat-deficient monkeys demonstrated a fresh corpus luteum. The adrenal and thyroid gland changes described by Alfin-Slater and Bernick ('58) in fat-deficient rats were also absent in the monkeys. The endocrine morphology of the present animals, however, must be evaluated with caution in view of the small numbers of intact sexually mature specimens.

Of interest was the absence of any of the various renal lesions that have been reported in rats (Borland and Jackson, '31; Panos and Finerty, '54; Aaes-Jorgensen et al., '56). The absence of microscopic hematuria and of gross renal lesions has been noted in dogs (Hansen and Wiese, '51).

The findings of hyperplastic bone marrow characterized principally by cells of the erythrocyte series is, perhaps, explicable on the basis of the increased erythrocyte fragility which has been reported in EFA-deficient rats (Macmillan and Sinclair, '58). Extramedullary erythropoesis (occasionally seen in normal laboratory *Cebus* monkeys) was prominent in one fat-deficient monkey. Day and Dinning[6] observed anemia in vitamin E-deficient rhesus monkeys. It was felt, however, that the absence of ceroid pigments in the tissues of fat-deficient monkeys, together with the low requirements for vitamin E in animals fed fat-free diets, probably excluded vitamin E deficiency as an explanation of the marrow hyperplasia.[7] Hematologic studies were not performed in the present study. Hansen and Wiese ('51) noted the absence of anemia in fat-deficient dogs, and Macmillan and Sinclair ('58) found a slight degree of polycythemia in fat-deficient rats.

The very low levels of linoleic and arachidonic acids and the increase in levels of monoenoic and eicosatrienoic acids in most tissues of the fat-deficient series of *Cebus* monkeys is consistent with the observations in many species. There was considerable difference in the resistance of different tissues and different classes of lipids to complete depletion of linoleic and arachidonic acid; the phospholipids as components of the "élément constant" were the most resistant to change.

Despite the supposed slow turnover of nervous tissue lipid there was clearly a difference in the concentration of different polyunsaturated fatty acids in control and fat-deficient monkeys. Analogous results were obtained by Rieckehoff et al. ('49) using rats.

The accumulation of cholesterol esters in the liver and adrenals of rats fed a fat-deficient diet or a diet high in saturated fatty acids (Alfin-Slater et al., '54) and in rabbits fed a fat-free diet (Diller et al., '61) was not characteristic of the fat-deficient monkeys. In fact, the cholesterol ester concentrations in liver and adrenals were lower in the fat-deficient than in the control monkeys. In agreement with observations in rats fed fat-free diets and man fed low-fat diets (Keys et al., '57) the serum total cholesterol and ester cholesterol concentration were also lower in the fat-deficient monkeys. If vitamin E deficiency was involved, in part, in determining the sterol concentrations of the fat-deficient monkeys it might be expected to elevate those concentrations (Jones et al., '57).

[6] Day, P. L., and J. S. Dinning 1956 Anemia in vitamin E-deficient monkeys. Federation Proc., *15*: 548 (abstract).

[7] Mason and Telford ('47) observed that ceroid pigmentation developed in rhesus monkeys after two years of feeding vitamin E-deficient diets. It is not known how early these pigments first appeared. These pigments were widely distributed in all types of muscle cells, and skeletal muscle showed dystrophic changes. None of these findings was present in the *Cebus* monkeys.

Although the aortic intimas of the fat-deficient monkeys had a higher concentration of ester cholesterol than did those of the control monkeys, intimal cholesterol esters could not be visualized under polarized light nor was there any histological evidence of atherosclerosis in the fat-deficient monkeys. Also absent from the fat-deficient monkeys were evidences of aortic medial calcification as reported by Hill et al. ('57) in young EFA-deficient swine. There is, therefore, little support in the studies of the *Cebus* monkey for the suggestion that EFA deficiency is responsible for abnormal cholesterol concentrations or for the acceleration of the development of atherosclerosis.

SUMMARY

Diets free of fat were fed to 9 *Cebus* monkeys for periods ranging from 8 to 22 months, and diets containing 8% by weight as corn oil were fed to 4 *Cebus* monkeys. Differences in fatty acid composition and cholesterol concentrations in a wide variety of tissues were determined and their significance discussed. A detailed histological examination of the monkeys was also made. Many of the biochemical and some of the histological changes characteristic of fat deficiency in rats were observed in the *Cebus* monkeys. The accumulation of increased concentrations of cholesterol esters in the liver and adrenals, which is characteristic of fat deficiency in rats, was not observed in the *Cebus* monkeys fed diets devoid of fat.

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The Free and Ester Sterol Content of Various Foodstuffs[1]

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The cholesterol content of various foodstuffs has been the subject of a number of compilations, the most complete being those of Okey ('45), Lange ('50), Pihl ('52) and Hayes and Rose ('57). In every case the total cholesterol content was reported.

Recent work by Swell et al. ('58a, b, '59) supports the concept that free cholesterol from the intestine is mixed with a free cholesterol pool in the intestinal mucosa prior to esterification and transfer to the lymph. Favarger ('52) fed rats cholesteryl esters labeled in the sterol and found evidence of hydrolysis prior to absorption. Using serum cholesterol elevation as an indicator of absorption it has been demonstrated that ester cholesterol is absorbed more slowly than free cholesterol in the rat (Swell et al., '55; Best and Duncan, '58) and the chicken (Peterson et al., '54).

In view of these reports we felt it would be of interest to determine the free and ester sterol content of various foodstuffs. A somewhat similar study had been carried out by Kaucher et al. ('43) but they used a limited number of samples and their results are based on dry weight which make rapid conversion to percentages in the original sample difficult.

METHODS

Samples of solids were extracted with chloroform-methanol 2:1 and dried aliquots were assayed for total cholesterol using Mann's ('61) modification of the ferric chloride method of Zlatkis et al. ('53). Another aliquot was treated with digitonin and the precipitated complex assayed by the same method. Ester cholesterol value were calculated by difference. Liquids were either extracted or diluted with the same solvents prior to analysis.

Determinations of the phytosterol content of foods have been carried out using gravimetric methods. (Steuart, '23; Hadorn and Jungkunz, '54). Other sources of information are compilations which also derive their data from gravimetric analysis (Kaufmann, '50; Lange, '50). Inasmuch as the principal sterols of most edible oils are sitosterol, stigmasterol or other minor sterols—all of which have the 3 hydroxy, 5–6 double bond system largely responsible for the chromogenic response of cholesterol to acidic reagents—we attempted to carry out the same colorimetric analysis using pure β-sitosterol as the standard. In figure 1 are shown the absorption curves of the chromogens from cholesterol and β-sitosterol and it is seen that with both sterols there is a maximum at 560 mμ.

The difference in extinction coefficient between cholesterol and sitosterol has also been observed by Breiskorn and Herrig ('59). They also found that stigmasterol and ergosterol reacted in a fashion similar to that of β-sitosterol, indicating a small, but significant effect for differences in the cholesterol side chain. Recent reports of the absorption maximum of desmosterol (Δ^{24}-cholesterol) (Avigan et al., '60) suggest the position of side chain unsaturation may also be a factor in color development. Standard curves for the two sterols (cholesterol and sitosterol) are shown in figure 2.

Most of the oils and all of the foods used were purchased in local markets.[2]

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[1] Supported in part by a grant H-3299 from the National Institutes of Health, U. S. Public Health Service.

[2] The soy and safflower oil were obtained from the Pacific Vegetable Oil Company, San Francisco.

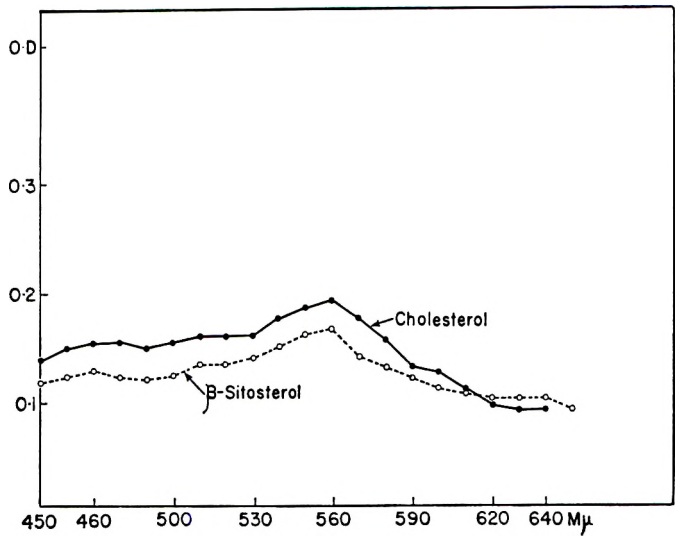

Fig. 1 Absorption curves of H_2SO_4–HOAc–$FeCl_3$ chromogens for cholesterol and β-sitosterol.

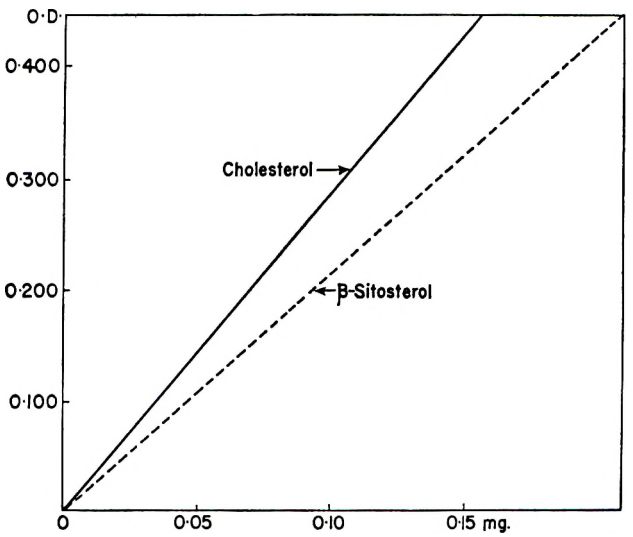

Fig. 2 Standard curves for cholesterol and β-sitosterol.

RESULTS AND DISCUSSION

The total cholesterol contents of the foods tabulated in table 1 are comparable to those reported by others, as are the free/ester cholesterol ratios. The observation that most meats contain appreciably less free cholesterol than most dairy foods is of interest here. If the extent of esterification of dietary cholesterol in man has an appreciable effect on cholesterol absorption and serum cholesterol levels, it would appear that per gram cholesterol ingestion of some dairy foods and eggs should cause higher cholesterol levels than meats. Data summarized by Cook ('58b) indicate that free cholesterol (crystalline) or egg yolk cholesterol is indeed absorbed to a considerable extent. The finding that cooking reduces the cholesterol content of meat confirms the report of Lobanov et al. ('58) and raises a point to be considered in assessing dietary intake.

The vegetable oil sterol levels (table 2) are in general agreement with values given

TABLE 1

Free and ester cholesterol content of various foods (mg/100 gm wet weight)

	Total	Free	Ester	% Ester
Meats				
Beef, ground	116	31	85	73
Beef, liver	262	136	126	48
Beef, steak, raw	114	47	67	58
Beef, cooked	62	31	31	50
Veal	85	80	5	6
Lamb	83	38	45	54
Lamb, liver	118	50	68	57
Bacon	215	61	154	71
Ham	126	29	97	77
Pork	98	27	71	72
Fowl				
Chicken	93	28	65	69
Chicken, liver	200	79	121	60
Turkey	110	50	60	54
Fish and sea food				
Clams	122	113	9	7
Cod	43	34	9	21
Flounder	41	22	19	46
Haddock	45	27	18	40
Halibut	34	26	8	23
Oyster	112	62	50	44
Salmon	65	57	8	14
Shrimp	138	127	11	8
Tuna	52	46	6	11
Dairy products				
Butter	187	85	102	54
Cheese, American	173	144	29	11
Cheese, bleu	174	156	18	10
Cheese, cream	140	118	22	17
Cheese, Swiss	150	150	—	—
Cream, thin	40	22	18	45
Cream, thick	140	49	91	65
Milk	28	28	—	—
Eggs	1862	1484	356	19
Lard	143	74	69	48

TABLE 2

Free and ester sterol of various vegetable fats (measured as sitosterol, mg/100 gm)

	Total	Free	Ester	% Ester
Corn oil, Mazola	1443	1159	284	12
Corn oil, Staley	861	767	94	11
Cottonseed oil, Ideal	499	357	42	8
Cottonseed oil, Wesson	576	507	69	12
Crisco	313	313	—	—
Margarine, Blue Bonnet	279	121	158	56
Margarine, Fleischmann's	165	67	98	59
Olive oil, Pompeiian	396	396	—	—
Peanut, Planters	321	309	12	4
Safflower, Pacific Vegetable	343	285	58	17
Soybean, Pacific Vegetable	990	802	188	19

by authors already cited, as well as those summarized by Cook ('58a). There are a few cases (olive oil, cottonseed oil) where the colorimetrically determined sterol levels are higher than the literature values. This can be explained, in part, by the relative lack of specificity of the Liebermann-Buchard reagent, which would give colors with other unsaturated compounds and by the possible presence of small amounts of sterols other than sitosterol or stigmasterol which could react with greater intensity. These differences would not affect the free/ester sterol ratio as determined by us. Of interest here are the relatively low levels of ester "phytosterol" in all but the margarine samples. As Best ('58) and Peterson et al. ('54) have shown, sitosteryl esters are less effective in blocking cholesterol absorption than is free sitosterol. Thus in this case the preponderance of free sterol may be beneficial.

SUMMARY

The free and ester cholesterol content of various foodstuffs has been determined. In general most of the cholesterol in meat is in the ester form (50 to 70%); in fish and sea food most of the cholesterol is present in the free form (55 to 95%). Butter and cream have equal quantities of free and ester cholesterol, whereas less than 20% of the cholesterol present in eggs and cheeses is in the esterified form.

The ferric chloride modification of the Liebermann-Burchard reagent used for cholesterol analysis was found to be equally useful for determination of β-sitosterol. Using this reagent the free and ester sterol content of various edible oils was determined. In two samples of margarine there were equivalent amounts of free and ester sterol. In the other fats analyzed, most of the sterol was present in the free form (80 to 100%).

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Relationship of Pancreatic Enzyme Secretion to Growth Inhibition in Rats Fed Soybean Trypsin Inhibitor

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The superior biological protein quality of properly heated soybean meal as compared with that of unheated soybean meal has been known for nearly a half century (Osborne and Mendel, '17); and numerous investigations have been conducted to determine possible mechanisms by which this improvement in protein availability is achieved.

With the discovery of a heat labile trypsin inhibitor in soybean (Ham and Sandstedt, '44; and Bowman, '44), attention was directed to the role of this factor in lowering the quality of the soybean protein. Animal studies showed that crude antitrypsin preparations from soybeans retarded growth in rats (Borchers et al., '48) and chicks (Ham et al., '45). Since the degree of inhibition seemed to be related to the amount of trypsin inhibitor in the meal, it was proposed that the growth depressing action of the trypsin inhibitor was due to a delayed release of methionine resulting from depressed intestinal proteolysis (Melnick et al., '46). Other reports, however, (Klose et al., '48; Westfall et al., '48) indicated that trypsin inhibitor exerted its growth retarding effects when fed with predigested proteins. Therefore, antiproteolytic activity could not account entirely for the adverse effect on growth. Liener ('53) successfully isolated a protein from raw soybean, which he has since called soybean hemagglutinin (Liener, '58). This material was closely associated with the trypsin inhibitor during isolation, and when fed to rats, seemed to specifically depress food intake. From his results, Liener concluded that the poor growth obtained with raw soybean flour or trypsin inhibitor concentrates was a function of the depression of food intake produced by soybean hemagglutinin and of the antiproteolytic or, possibly, some other characteristic of the antitrypsin.

Recently, Lyman and Lepkovsky ('57) have shown that rats allowed a single feeding of either raw soybean meal diet or heated soybean meal supplemented with crude or crystalline soybean trypsin inhibitor secreted large quantities of digestive enzyme into the small intestine. Although the proteolytic enzyme activity in the intestinal tract of these animals was excessive, intestinal nitrogen remained consistently higher than that in the controls (Lyman, '57). The results suggested that, in the rat, the apparent poor absorption of nitrogen was due to the excessive enzyme secretions and that growth inhibition produced by trypsin inhibitor might be related to the excretion of this nitrogen. The experiments were of an acute nature, however, and involved only a single feeding of raw soybean or trypsin inhibitor supplemented diet. Therefore, the following experiments were performed, with rats fed crude trypsin inhibitor or raw soybean diets, to investigate whether a similar enzyme response maintained over an extended period could be related to growth inhibition, or whether intestinal proteolysis was reduced by a combination of inhibitor activity and exhaustion or adaptation of the pancreas.

EXPERIMENTAL

Weanling female rats of the Long-Evans strain were fed unextracted soybean meal diets, the composition of which is given

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in table 1. The whole soybean meals were added to the diet so as to provide 12% of protein[2] (6.25 × N). The heated soybean diet was supplemented with crude soybean trypsin inhibitor (SBTI) so that its trypsin inhibiting activity was comparable with that of the raw soybean diets when assayed *in vitro*.

The procedure used for the preparation of the crude soybean trypsin inhibitor concentrate was a modification (Lyman and Wilcox, '60) of a method described by Borchers et al. ('48). Material prepared in this manner had about 25 times the trypsin inhibiting activity of the whole raw soybean meal. For the animals fed ad libitum, food consumption and body weights were recorded twice weekly. The average amount of diet eaten by the experimental animals was determined daily and all the animals in the pair-fed control groups were fed that amount of heated soybean control diet. Overnight fecal excretion was collected weekly from each group. The feces were lyophilized, weighed and ground for analyses.

Each week during the three-week experiment, rats were sacrificed from each group. The animals were fasted the night before autopsy, and on the day of an experiment they were allowed access to their food for three hours. During this time, 3 to 5 gm of diet generally were consumed. It was found, however, that consumption of only 1 gm was sufficient to elicit a pancreatic response.

The animals were decapitated under sodium pentobarbital anesthesia and the pancreas and intestinal contents obtained as described by Lyman and Lepkovsky ('57). Pulverized samples, weighed and homogenized in distilled water, were used for the enzyme assays. The methods for determining proteolytic activity in the pancreas and intestinal material have been described (Lyman and Lepkovsky, '57). Results for protease activity[3] are expressed as milliequivalents of tyrosine released per 10 minutes of incubation at 37°C. Nitro-

[2] This level of protein was considered to give maximum utilization of nitrogen for growth (Liener et al., '49).

[3] The trypsinogen of the pancreas was activated by a crude preparation of enterokinase, Viodenum (Viobin Corp., Monticello, Illinois). Unactivated pancreas tissue showed no protease activity.

TABLE 1

Composition of soybean[1] rations

Component	Heated soybean	Heated soybean + trypsin inhibitor	Raw soybean	Heated soybean + methionine	Heated soybean + trypsin inhibitor + methionine	Raw soybean + methionine
	%	%	%	%	%	%
Heated soybeans[2]	35.0	35.0	—	35.0	35.0	—
Raw soybeans	—	—	35.0	—	—	35.0
DL-Methionine	—	—	—	0.5	0.5	0.5
Crude trypsin inhibitor	—	1.4	—	—	1.4	—
Powdered sucrose	55.0	53.6	55.0	54.5	53.1	54.5
Cottonseed oil	4.0	4.0	4.0	4.0	4.0	4.0
Fortified oil[3]	1.0	1.0	1.0	1.0	1.0	1.0
Salts[4]	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin mix[5]	1.0	1.0	1.0	1.0	1.0	1.0
	100.0	100.0	100.0	100.0	100.0	100.0

[1] Clark soybeans purchased from C. M. Volkman and Company, San Francisco. Heated meal analyzed (in per cent): protein, 33.0; water, 5.70; fat, 24.0; and raw meal: protein, 34.2; water, 6.49; and fat, 23.0.

[2] The whole soybean was autoclaved for 15 minutes at 15 pounds pressure, then dried at room temperature, and ground. The meal obtained was fed without extraction of the fat.

[3] Cottonseed oil fortified to provide: vitamin A, 1700 I.U.; vitamin D, 100 I.U.; α-tocopheryl acetate, 6.66 mg; and menadione, 0.5 mg per 100 gm of diet.

[4] Hubbel et al. ('37), Salt Mixture H.M.W., obtained from Nutritional Biochemicals, Cleveland.

[5] Compounded to provide per kg of diet in milligrams: thiamine, 2.0; riboflavin, 3.0; pyridoxine, 2.5; pantothenic acid, 20.0; inositol, 100; biotin, 0.1; folic acid, 0.2; niacinamide, 10.0; vitamin B_{12} (as 0.1% triturate), 20.0; and choline chloride, 500.

gen analyses were made using all-glass, semimicroKjeldahl equipment. Copper sulfate was used as the catalyst during the digestion.

In order to determine the digestibility of the diets and the relative amounts of nitrogen occurring in the digestive tract, diets marked with 1% chromic oxide were fed. The method of Dansky and Hill ('52) was used to determine the amount of chromic oxide incorporated into the diets and the chromic oxide appearing in the intestinal contents and feces.

RESULTS

The results in table 2 show the food consumption, weight gains and food utilization of the rats after receiving their respective diets for three weeks.

Soybean trypsin inhibitor added to the heated soybean diets depressed growth and food efficiency when compared with either the pair-fed or ad libitum control groups. Nearly one third of the growth depression appeared to be the result of the reduced food intake. Supplementation of the same diets with methionine improved both food utilization and growth considerably but did not restore either quite to normal. The raw soybean diets, although possessing trypsin inhibitor activity equivalent to the SBTI-supplemented diets, produced a much more pronounced growth depression and reduction in food utilization. With these diets, methionine improved growth, but the response was far less than that observed with the SBTI diet. Inhibition of food intake was much greater in the rats fed raw soybean. This alone, however, could not account for the severity of the growth reduction, since methionine-supplemented animals still grew only half as well as their pair-fed controls, and food utilization remained greatly impaired.

To determine whether SBTI interfered with intestinal proteolysis, groups of rats were sacrificed weekly, and protease analyses were made on the pancreas, intestinal contents and feces. The results are shown in figure 1. In the groups fed SBTI or raw soybean diets, but no supplementary methionine, the pancreatic protease activities remained similar and quite constant during the experimental period. The protease activity in the small intestine of these groups, however, was increased to

TABLE 2

Growth and food consumption of rats fed raw soybean- or trypsin inhibitor-supplemented heated soybean diets with and without additional methionine

Diet	No. of rats	3-Week food consumption	3-Week weight gain	Food efficiency
		gm	*gm*	*gm gain/ gm food consumed*
1 Heated soybean ad libitum	8	246 ± 13[1]	78 ± 4	0.32 ± 0.01
2 Heated soybean + trypsin inhibitor ad libitum	8	205 ± 13	48 ± 5	0.23 ± 0.01
3 Heated soybean restricted to group 2	4	216 —	67 ± 1	0.31 —
4 Heated soybean + methionine ad libitum	8	254 ± 8	96 ± 5	0.38 ± 0.01
5 Heated soybean + methionine + trypsin inhibitor ad libitum	8	226 ± 9	71 ± 2	0.31 ± 0.01
6 Heated soybean + methionine restricted to group 5	4	219 —	82 ± 1	0.38 —
7 Raw soybean ad libitum	8	146 ± 12	5 ± 6	0.03 ± 0.02
8 Heated soybean restricted to group 7	4	160 —	44 ± 1	0.28 —
9 Raw soybean + methionine ad libitum	8	149 ± 7	22 ± 3	0.15 ± 0.02
10 Heated soybean + methionine restricted to group 9	4	143 —	46 ± 3	0.32 —

[1] Standard error of the mean.

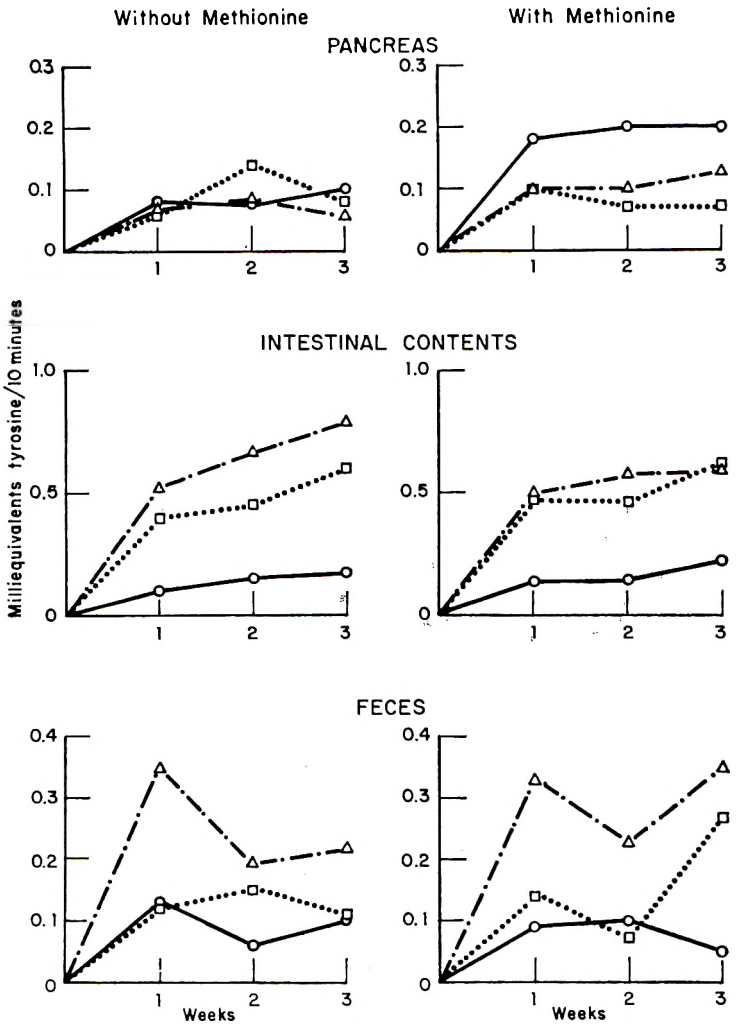

Fig. 1 The effect of raw soybean or SBTI-supplemented heated soybean diets with and without additional methionine on proteolytic enzyme activity in the pancreas, intestinal contents and feces of rats. Results are plotted as milliequivalents of tyrosine per 100 gm of body weight in the case of the pancreases. Intestinal contents and feces are reported as milliequivalents of tyrosine per total amount of material obtained. Key: ○—○, heated soybean control diets; △–·–△, heated soybean diets + crude soybean trypsin inhibitor; □ · · · · □, raw soybean diets.

three to 5 times that of the controls, and remained elevated throughout the experiment. There was no evidence of depletion or exhaustion of pancreatic protease. Protease analyses of the feces were not too satisfactory because of the variability in the amounts excreted, but there was some evidence that a part of the enzyme was excreted intact. Methionine had little influence on the amount of enzyme secreted into the intestine or excreted with the feces. One striking effect of this amino acid seemed to be its maintaining of a much higher reserve of proteolytic enzyme activity in those pancreases not subjected to SBTI stimulation. As a check on the protease analyses, lipase determinations were also made on all the samples. Since the excretion pattern of this enzyme was almost identical with that of the protease,

the results have not been included here, but are available elsewhere.[4]

Results of the present study show no evidence that a deficiency of intestinal proteolytic activity was produced when SBTI or raw soybean was fed to rats. From the excessive quantities of active protease noted in the intestine of these animals, it might reasonably be expected that protein utilization would be improved. When the nitrogen in the intestinal contents was determined, however, total nitrogen in the small intestine of SBTI-supplemented and raw soybean-fed rats remained consistently higher than that in the control groups, suggesting that the dietary nitrogen was poorly utilized (fig. 2). Additional

[4] Haines, P. C. 1960 The relationship of pancreatic enzyme secretion to growth inhibition and nitrogen excretion in growing rats fed soybean trypsin inhibitor. Thesis, University of California, Berkeley, 1960.

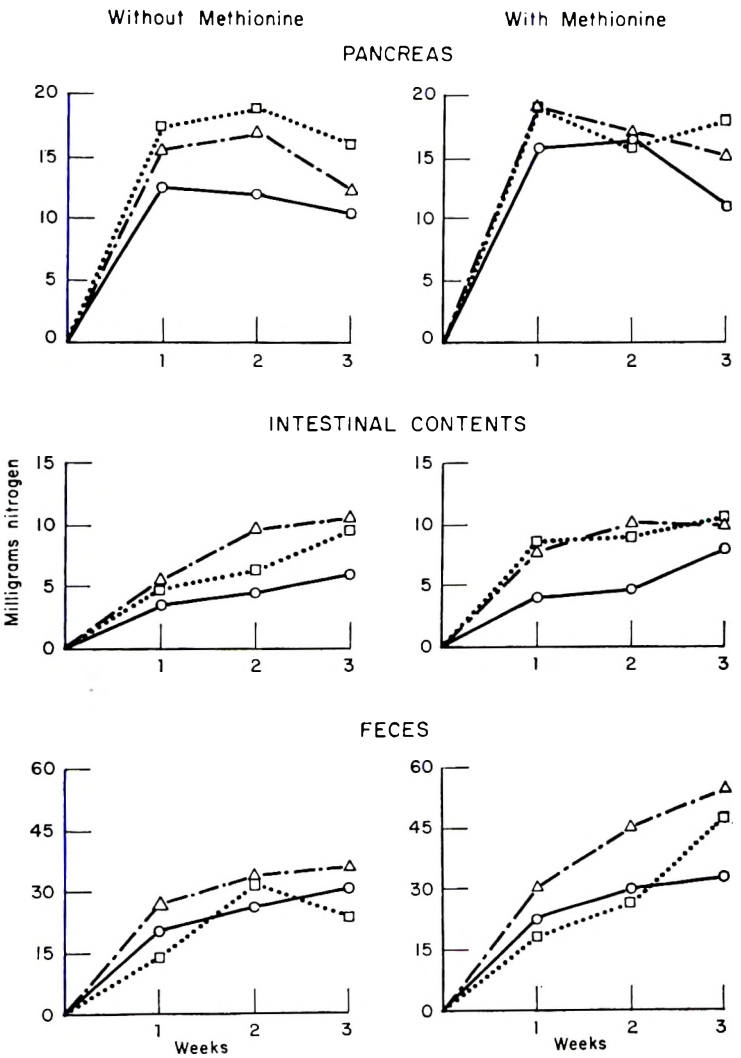

Fig. 2 The effect of raw soybean or SBTI-supplemented heated soybean diets with and without additional methionine on pancreatic, intestinal and fecal nitrogen of rats. Pancreas nitrogen is plotted as milligrams of nitrogen per 100 gm of body weight. Intestinal and fecal nitrogens are reported as total milligrams nitrogen in material obtained. Key: ○—○, heated soybean control diet; △– · –△, heated soybean + SBTI; □ · · · · □, raw soybean diet.

methionine added to the diets did not modify this effect. Fecal nitrogen analyses were inconclusive except that SBTI-fed rats maintained a high nitrogen excretion as they had with protease activity. Pancreatic nitrogen seemed to be highest in the rats fed SBTI and raw soybean diets. The probable explanation for this is that the pancreases of these animals were considerably enlarged when compared with those of the controls. Methionine had no effect on this hypertrophy. Booth et al. ('60) have demonstrated similar pancreatic enlargement in rats fed raw soybean diets for 35 days. In the present experiments, the effect was noticeable as early as one week after starting the diet and apparently represents an adaptive response by the pancreas to the continual secretory stimulus produced by SBTI.

Results of the digestibility studies are presented in table 3. It was felt that the digestibility data are best expressed as apparent coefficients of nitrogen absorption, since they represent the net absorption of dietary as well as endogenous nitrogen from the alimentary tract. As might be expected from the intestinal nitrogen data, SBTI or raw soybean significantly reduced the apparent coefficients of nitrogen absorption obtained from the small intestine. The coefficients obtained from analyses of the feces were much higher and the differences between the groups much less, although SBTI or raw soybean still reduced the values below that of the control group. The higher absorption coefficients obtained from the feces would indicate that part of the intestinal nitrogen was absorbed prior to excretion, although it is doubtful that such nitrogen could be used efficiently for growth purposes. Rats fed the raw soybean meal showed the largest variability and the lowest apparent nitrogen utilization from the intestine. A few of the coefficients calculated were actually negative. This was similar to results obtained by Carroll et al. ('52) who interpreted them as indicating an increase in endogenous nitrogen secretion.

DISCUSSION

The results of this study confirm and extend those obtained previously with a single feeding of soybean trypsin inhibitor or raw soybean meal (Lyman, '57). When fed daily as part of the diet, SBTI depressed growth and reduced the efficiency of food utilization. Neither effect could be attributed to any inactivation or loss of intestinal protease activity resulting from pancreatic insufficiency or antiproteolytic action of the inhibitor. The results, therefore, cannot be explained on the basis of an inhibited intestinal protein hydrolysis.

Recently, Borchers et al.[5] showed that growth of rats fed raw soybean meal can be restored to normal by the addition of 4 amino acids, methionine, valine, threonine and tyrosine. Booth et al. ('60) confirmed this work and, in addition, showed that the pancreases of rats fed raw soybean were hypertrophic and exhibited distinct morphological changes. On the basis of their results and those obtained previously by Lyman ('57), Booth et al. hypothesized that growth depression produced by raw soybean might have resulted from the excessive pancreatic enzyme secretions and the loss of endogenous pro-

[5] Borchers, R. 1959 Tyrosine stimulates growth on raw soybean rations. Federation Proc., *18:* 517 (abstract).

TABLE 3

Apparent coefficients of nitrogen absorption for rats fed raw or SBTI-supplemented heated soybean diets marked with chromic oxide

Diet	No. of rats	Intestinal contents	Feces
Heated soybean	9	64.5 ± 1.6[1]	82.1 ± 1.0
Heated soybean + trypsin	10	45.7 ± 2.8[2]	73.1 ± 0.8[2]
Raw soybean	9	22.6 ± 16.8[3]	62.6 ± 6.7[3]

[1] Standard error of the mean.

[2] Significantly lower ($P < 0.01$) than the heated soybean group (as compared by t test, Snedecor ('46)).

[3] Not significantly lower ($P < 0.1$) than heated soybean plus trypsin due to wide variation in values.

tein which ultimately produced deficiencies of certain amino acids.

The present results with the crude SBTI are consistent with this concept. The addition of a relatively small amount of crude SBTI to a readily available protein source resulted in an increase in the apparent unabsorbed nitrogen in the small intestine, yet an excess of proteolytic enzyme activity was always present. It would not be expected that a protein should be any less digestible under such conditions. Therefore, the excess intestinal nitrogen must have been derived from the endogenous pancreatic enzyme secretions.[6] The improvement in growth and protein utilization obtained with additional methionine would be anticipated, since at the 12% protein level used in these experiments, any additional loss of cystine or methionine, the most growth limiting amino acids in soybean protein, would create a demand for methionine greater than the diet could provide. Valine or threonine are, perhaps, the next growth limiting amino acids at this level of protein. Borchers ('58) demonstrated that 40% of raw soybean meal in diets supplemented with methionine increased the growth of rats to that produced by an equivalent level of methionine-supplemented heated soybean meal. The author concluded that no toxic substances were limiting growth in rats fed raw soybean. In view of the present results, it would appear that the additional protein plus the methionine provided by Borchers' diet made available amounts of essential amino acids that were sufficient to offset any losses induced by SBTI.

Both the SBTI-supplemented heated soybean and the raw soybean diets produced nearly the same pancreatic secretory response, yet growth depression was most severe in the raw soybean-fed rats. The major difference between the two groups was the depressed food intake in the latter animals. The cause of this effect is not fully understood, although Liener ('53) attributes it to soybean hemagglutinin. Borchers' studies, however, suggest that the provision of the right amino acids is more important and will counteract the reduction in food intake and subsequent growth depression. In the present studies, the combination of a low food intake and a relatively constant loss of endogenous nitrogen may have intensified certain amino acid insufficiencies. It would seem that soybean hemagglutinin initially inhibits food intake, with SBTI contributing a secondary amino acid deficit which then reinforces the original food intake inhibition.

A report by Lepkovsky et al. ('60) indicated that chicks fed raw soybean diets excrete a higher protease activity in their feces than their controls fed heated soybean. These results might indicate that the rat and the chick were behaving similarly in their response to SBTI. A recent publication (Alumot and Nitsan, '61), however, clearly demonstrated that intestinal proteolysis in chicks fed raw soybean diets was almost completely inhibited by the SBTI for three weeks and the activity did not return to normal until 6 weeks. Therefore, for the young, growing chick, inhibition of intestinal proteolysis is, undoubtedly, a major factor producing the poor growth in birds fed raw soybeans. Rats, fed raw soybean or SBTI and heated soybean protein by stomach tube, show an increased intestinal protease activity as early as a half-hour after feeding.[7] Therefore, the physiological response to SBTI in the two species is apparently quite different. Whereas in the rat the inhibitor causes an immediate outpouring of enzymes from the pancreas, in the chicken this response does not occur for many weeks.

SUMMARY

Rats were fed either heated soybean diets supplemented with soybean trypsin inhibitor (SBTI) or raw soybean diets having equivalent protein and trypsin inhibitor activity for three weeks. During the experiment, growth, intestinal protease activity, food utilization, and nitrogen absorption were determined.

The SBTI-supplemented diet depressed growth and reduced food utilization, but

[6] Calculations based on the average amount of intestinal contents (170 mg), coefficients of absorption (45.7%) and amount of inhibitor in the ration (1.4%) show that if none of the crude inhibitor was absorbed, it would contribute only 0.7 mg of nitrogen to the intestinal nitrogen.

[7] Lyman, R. L., unpublished data.

with considerably less severity than did the raw soybean diet. Pair feeding, to equalize food intake, indicated that the growth depression produced by the raw soybean resulted largely from a depressed food intake. Food consumption was much less depressed by the SBTI diets. Analyses of the intestinal contents revealed that rats fed SBTI or raw soybean maintained a high level of intestinal protease activity, yet the animals appeared to absorb less nitrogen than the control rats.

It was concluded that growth depression produced in the rat by raw soybean results from a combination of restricted food intake and the effects of trypsin inhibitor, which acts, not by inhibiting protein hydrolysis, but by stimulating the pancreas to secrete large quantities of enzyme protein which are ultimately lost to the animal. The effect of this is to create partial deficiencies of the most growth limiting amino acids in the soybean protein.

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Endogenous Nitrogen Secretions into the Digestive Tract

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Recent reports on the magnitude of endogenous nitrogen secretions into the gastrointestinal tract have been conflicting. Dreisbach and Nasset ('54) noted that the amount of nitrogen recovered from the gastrointestinal tracts of rats fed 18% casein diets was equal to or greater than the amount of nitrogen ingested. Geiger et al. ('58), however, consistently recovered less nitrogen from rat gastrointestinal tracts than had been fed in skim milk preparations. Also, Peraino et al. ('59) found that when they fed nitrogen at a range of zero to 350 mg, rat intestinal nitrogen content remained low.

Several experimental techniques have been used in studies of the magnitude and importance of the endogenous nitrogen secretions. For example, by amino acid analysis, Nasset et al. ('55; '58) demonstrated that the blending of endogenous proteins with various dietary proteins tended to provide relatively similar amino acid mixtures for absorption from the small intestinal contents of dogs. Post-feeding plasma amino acid levels, however, have been correlated, somewhat, with the amino acid content of the dietary protein (Longenecker et al., '59; Guggenheim et al., '60). Nevertheless, this correlation could exist even though endogenous proteins were blended with dietary proteins in the intestine. Boyne et al. ('56), on finding in sheep that the ratio of total nitrogen to insoluble ash greatly increased between the abomasum and the proximal part of the small intestine, postulated the occurrence of a considerable addition of nitrogenous compounds to the intestinal contents. Meyer et al. ('59), using nitrogen-lignin ratios, calculated that nitrogen increased 175% on passing from the abomasum through the small intestine of sheep.

The experiment reported herein made use of an inert indicator in the feed in a further attempt to study the magnitude of endogenous nitrogen secretions into the digestive tract on lower levels of dietary protein.

EXPERIMENTAL

Male Sprague-Dawley rats, ranging in weight from 150 to 200 gm, were fed one of 5 levels of whole-egg protein.[1] The protein constituted zero, 5, 10, 15 or 20% of the ration and was added at the expense of the sucrose in the diet.[2] The remainder of the rations consisted of the following percentage composition: chromic oxide, 1; cellulose, 5; vitamins,[3] 1; salts,[4] 4; and cottonseed oil,[5] 5. Thirty rats were assigned to each of the 5 rations and were trained to eat in one-hour periods, 12 hours apart.

The rats were supplied with the experimental rations for one week. During the last feeding period of the experiment, rats fed protein-free diets consumed, on the average, 4 gm of ration. Food consumption for the other groups of rats ranged between 6.3 and 7.0 gm, the consumption of 5 and 20% protein diets, respectively. At this time 6 rats from each ration were sacrificed just prior to feeding and at the

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[1] Egg protein was prepared by exhaustive hexane extraction of whole, steam-cooked, dried egg (shells not included).

[2] The protein provided zero to 26 mg of nitrogen per gm of ration depending on the level at which it was fed.

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

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

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

following hours after the initiation of feeding: 1, 2.5, 5 and 8. The gastrointestinal tracts were removed after ligation at the esophageal, pyloric and ileocaecal sphincters. The contents of the stomach and the small intestine were dried separately on cellulose. After drying, each mixture of cellulose and contents was ground. Samples from each mixture were taken for use in analysis for nitrogen and chromic oxide. Nitrogen was determined by the Kjeldahl method. The chromic oxide samples were analyzed according to the method described by Kimura et al. ('57). Commencing 4 days prior to killing, fecal collections were taken from each rat and analyzed for nitrogen and chromic oxide.

RESULTS AND DISCUSSION

Changes in the dry matter and nitrogen content of the stomach with time are shown in table 1 where gastric dry matter and nitrogen content are tabulated as percentage of the dry matter and nitrogen consumed. Each figure represents an average of the results from 6 rats for each ration and for each observed time period. A larger portion of the total gastric nitrogen content of rats receiving low-protein rations appeared to be of endogenous origin. Corrections for this endogenous portion were made for all groups of rats at all time periods by subtracting the average gastric nitrogen content of rats fed a protein-free diet from the average gastric nitrogen content of protein-fed rats. These corrections are not completely valid since endogenous nitrogen secretions into the stomach may increase with dietary protein. They do clarify, however, the emptying pattern of gastric nitrogen. In table 1 it is shown that approximately 40 to 50% of the gastric contents had emptied by 2.5 hours after the initiation of feeding. The remainder of the contents emptied during the next 9.5 hours. Rosenthal and Nasset ('58) stated that the stomach emptied its contents in an exponential fashion during the first 5 hours after feeding. Rogers et al. ('60) found that the shapes of stomach-emptying curves varied from almost exponential to nearly linear depending on the nature of the diet. Lepkovsky et al. ('57) noted that 50% of the ingested food was digested in approximately 2.5 hours

TABLE 1

Nitrogen and dry matter content of the stomach

% Protein fed	Hours after feeding									
	1		2.5		5		8		12	
	Nitrogen[1]	Dry matter[2]	Nitrogen	Dry matter	Nitrogen	Dry matter	Nitrogen	Dry matter	Nitrogen	Dry matter
0		67.7		45.1		31.7		13.9		2.8
5	76.3	82.1	56.7	57.3	43.4	34.1	18.6	11.7	9.0	3.4
10	69.2	70.3	61.0	62.4	33.0	28.6	18.5	12.3	1.0	1.4
15	83.3	86.5	49.5	49.6	32.8	35.6	21.1	18.6	0.7	0.7
20	70.7	86.5	48.3	49.0	38.3	39.5	18.9	21.1	0.3	2.0

[1] Corrected for endogenous nitrogen secretions and calculated as percentage of nitrogen consumed.

[2] Calculated as percentage of dry matter consumed.

regardless of the amount eaten. Nitrogen and dry matter apparently left the stomach at the same rate during the course of this experiment. Variations between the nitrogen and dry matter content of the stomach were not consistent.

The dry matter and nitrogen content of the small intestine are plotted against time for each protein level fed in figures 1 and 2, respectively. No attempt was made to compensate for differences in food consumption between rats. Coefficients of correlation between intestinal dry matter content and food consumption were not significant. Lepkovsky et al. ('57) noted that the total solids of the intestinal contents varied little, although different amounts of food were eaten by different rats.

Immediately after feeding, the dry matter content of the small intestine rose sharply (fig. 1). About one hour after the initiation of feeding the dry matter content began to decline. Increases observed in the dry matter content of the small intestine after 2.5 hours were not as large as those noted one hour after the initiation of feeding.

Nitrogen accumulation patterns[6] for the various diets are presented in figure 2. They demonstrate that the nitrogen content of the small intestine varied considerably with time. Also, nitrogen content rose as dietary protein was elevated. As with dry matter, the nitrogen content of the small intestine increased immediately after feeding except in rats fed the protein-free ration. The sudden passage of gastric contents into the small intestine must have been chiefly responsible for this increase since a corresponding increase was not noted in rats fed the protein-free ration. Another generally sharper rise occurred at 2.5 hours, reaching a peak at 8 hours. This rise was noted for the protein-free ration as well as for the 4 protein rations. With the protein-free ration an elevated accumulation of endogenous nitrogen in the small intestine must have led to this rise. But, the magnitude of the nitrogen accumulation increased with dietary protein, up to the 15% level. Was this additional nitrogen accumulation due to elevated dietary accumulation or to augment accumulation of endogenous nitrogen—principally of digestive enzymes? (Part of the increase in nitrogen accumulation in rats fed 5% of protein over rats fed no protein might have been due to greater mucosal slough-off. The latter is affected mainly by changes in dry matter consumption or excretion [Meyer et al., '56].)

An elevated dietary accumulation could result from an increased rate of gastric release of food or a lowered rate of digestion, absorption or of passage through the small intestine. The former assumption is incorrect since 60 to 70% of the gastric contents were emptied by 5 hours after feeding. Only about 20% of the gastric contents entered the small intestine between 5 and 8 hours. Increases in slowly-digestible dietary protein probably would not account for the additional nitrogen accumulation. Whole-egg protein is quite digestible. If a portion of the protein were slowly digested, however, accumulation would have begun before 2.5 hours. Therefore, after 2.5 hours, the maximum amount of dietary nitrogen present in the small intestine might simply be represented by the difference between the small intestinal nitrogen content of the protein-free-fed rats at 2.5 hours and the protein-fed rats at 2.5 hours.

In addition, if the increased accumulation were due to a lowered absorption rate of food material, each milligram rise in nitrogen would cause the dry matter content of the small intestine to increase according to the factor—$6.25 \times 100/\%$ of dietary protein. This calculation is valid if nitrogen absorption does not lag significantly behind the absorption of other materials from the diet. In this manner, the expected increases in small intestinal dry matter were calculated from the observed increases in small intestinal nitrogen. The expected and observed increases in dry matter for the various pro-

[6] The patterns shown in figure 2 vary somewhat from those noted by Rogers et al. ('60) for several protein diets although observations for a protein-free diet were similar. Dissimilarities could be due to differences in dietary regime since these authors fed neither whole-egg protein nor cellulose, which could alter the pattern and the amount of the endogenous nitrogen secretions. Also zero time in their experiment represented the hour at which feeding terminated with no values given for the nitrogen content of the small intestine prior to feeding.

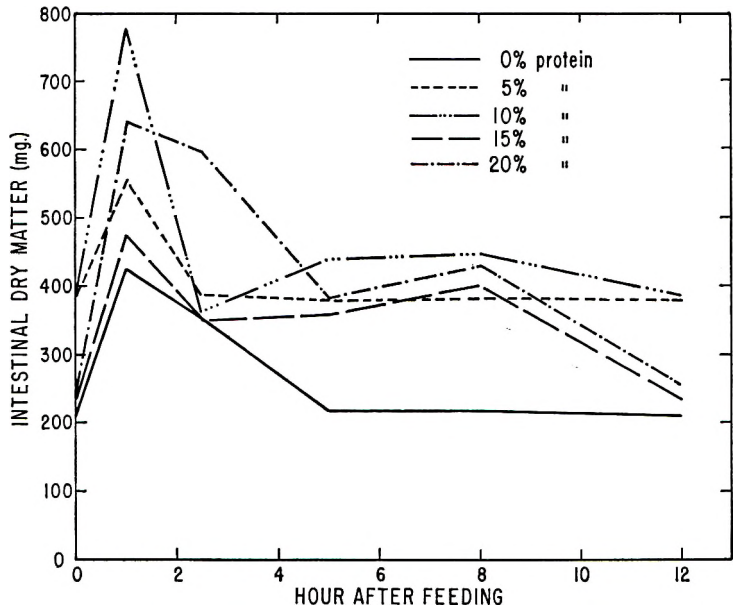

Fig. 1 Mean small intestinal dry matter content of all rats on each treatment.

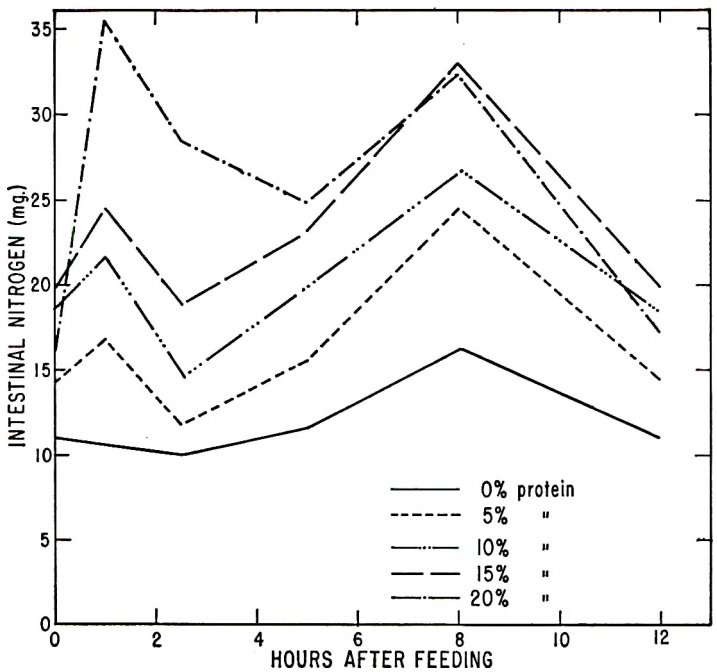

Fig. 2 Mean small intestinal nitrogen content of all rats on each treatment.

tein rations during the time intervals, zero to one and 2.5 to 8 hours, are presented in table 2. The figures shown therein demonstrate that the expected and observed values correspond more closely for the time interval, zero to one hour. The observed values for the time interval 2.5 to 8 hours, are consistently lower than the ex-

TABLE 2

Dry matter accumulation in the small intestine

% Protein fed	Nitrogen accumulation		Expected dry matter[1] accumulation		Observed dry matter accumulation	
	0–1[2]	2.5–8[2]	0–1	2.5–8	0–1	2.5–8
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
0	—	6.2	—	—	—	—
5	2.4	12.4	300	1550	175	0
10	2.7	11.6	169	725	395	81
15	4.6	15.0	192	626	240	49
20	18.4	7.2	576	225	380	50

[1] Calculated by multiplying nitrogen accumulation by 6.25 × 100% of dietary protein. This assumes the same ratio of nitrogen or protein to dry matter in the contents as in the diet.
[2] Time interval after the initiation of feeding; zero to 1 represents the feeding period.

pected values. Thus, the increased accumulation of small intestinal nitrogen during the first hour after the initiation of feeding seemed to be principally of dietary origin. The 8-hour peak elevation in nitrogen was probably not of dietary nature since small intestinal dry matter content did not increase accordingly. Gradual accumulations of slowly digestible enzymes, cellular breakdown products, and other nitrogenous compounds must have been responsible for this peak. Since nitrogenous accumulation at this time appeared to rise with the level of dietary protein, endogenous secretory accumulations must have risen also. Possibly the animal reaches its mamimum secreting ability at the 15% level of dietary protein since small intestinal nitrogen accumulation did not increase at 8 hours at the 20% level of the dietary protein. Another possible explanation exists for the small intestinal nitrogen accumulation on this level of dietary protein. An unproportionally large increase in intestinal nitrogen occurred during the feeding period (zero to 1 hour) with the 20% protein ration. Also, the observed dry matter increase at this time (table 2) was not as large as expected. Therefore, a larger portion of the total endogenous nitrogen might have been secreted earlier during the course of digestion of a 20% protein ration, and endogenous nitrogen secretions may increase still further with a 20% protein ration.

Mitchell et al. ('54) postulated that metabolic nitrogen excreted in the feces was independent of the level of dietary protein by showing that metabolic fecal nitrogen determined on a low-protein ration was the same as extrapolation of a series of nitrogen levels to zero. A regression line similar to that calculated by Mitchell by the method of least squares is shown in figure 3 where

$$Y = 1.31 + 0.0459X.$$

Y represents fecal nitrogen in grams per kilogram of food consumed, and X is equal to the percentage of protein in the diet. The figure, 1.31, obtained by extrapolating a series of protein levels to zero and representing the calculated amount of fecal nitrogen excreted when using a protein-free ration, is significantly lower than the actual figure (1.52) obtained by averaging the results from 30 rats fed a protein-free diet. This discrepancy can be accounted for by assuming that the minimum nitrogen excretion using whole-egg protein diets occurs at the 5% level of ingestion. The possibility remains that as protein ingestion rises above the 5% dietary level, excretion of both dietary and endogenous nitrogen may increase. The exact nature of the consistent rise of fecal nitrogen with dietary protein is questionable.

Other work has shown that the level of protein can affect the secretion of digestive enzymes. Sumi ('58) demonstrated a correlation between protein in the diet and promotion of the secretory function of the peptic cells. Gadzieva ('56) showed that the protein output of the intestinal secretions of dogs decreased when supplied with a low-protein diet. Nitrogen depletion with an inadequate protein diet can have a diminishing effect on enzymatic secretions into the digestive tract (Albanese, '59).

Estimations of the magnitude of the endogenous nitrogen secretions and mucosal

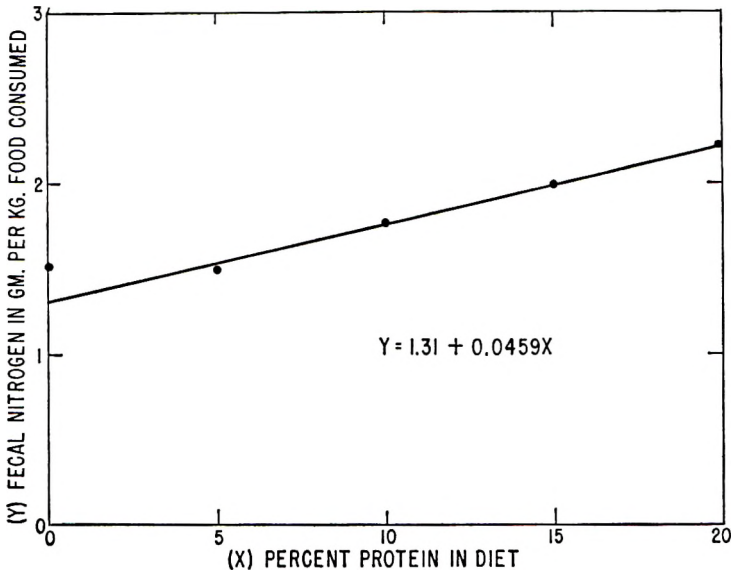

Fig. 3 The regression of the ratio of fecal nitrogen to food consumed on protein in the diet. Each point represents an average of the actual results obtained from 30 rats on each ration.

slough-off into the gastrointestinal tract can be made through the use of chromic oxide as an inert indicator. In this experiment chromic oxide appeared to lag behind other dietary material when passing from the stomach. Some chromic oxide passed from the stomach during the zero to 1 hour time interval. Chromic oxide passed most rapidly from the stomach after 5 hours. Thus, chromic oxide and nitrogen seemed to follow the same general pattern of variation in the intestine. If a protein-free diet is fed, the proportion of the endogenous nitrogen excreted in the feces can be calculated in the following manner:

$$\%\ \text{endogenous nitrogen excreted in feces} = 100\left(\frac{a}{b} \times \frac{c}{d}\right)$$ where

a = % Cr_2O_3 in small intestinal contents
b = % Cr_2O_3 in feces
c = % nitrogen in feces
d = % nitrogen in small intestinal contents.

Then, knowing the total nitrogen excretion, one can calculate the total endogenous nitrogen secretion and slough-off by the following formula:

$$\text{Endogenous nitrogen secreted and sloughed off} = \frac{\text{total nitrogen excretion}}{\%\ \text{endogenous nitrogen excreted in feces}}.$$

This calculation was made from the nitrogen-chromic oxide ratios obtained in the small intestines of rats fed the protein-free diet and killed at 8 hours. At this time chromic oxide and nitrogen were approaching their maximum values in the intestine.

An estimated 563 ± 147[7] mg of nitrogen were secreted or sloughed into the digestive tract of rats fed a protein-free diet over a period of 4 days (8 feedings). This value is equivalent to the amount of nitrogen consumed by rats fed a 10% level of dietary protein over a period of 4 days. This method of calculation underestimates the magnitude of endogenous nitrogen secretions and slough-off. Dry matter-chromic oxide ratios in small intestinal contents range far below the dietary dry matter-chromic oxide ratio—approximately 95. For rats fed the protein-free ration these intestinal ratios average 37.4 and 23.2 at one and 8 hours respectively. Nitrogen-chromic oxide ratios must also be somewhat low in the intestine, since the passage rate of chromic oxide through the small intestine is probably slower than the breakdown and reabsorption rates of endogenous protein. For this reason, use of the chromic oxide indicator tends to

[7] Standard error of the mean.

lead to underestimations of the nitrogen accumulation in small intestinal contents.

Use of this same formula and figures 2 and 3 allows a calculation of endogenous nitrogen secretion on protein rations assuming (1) the maximum amount of dietary nitrogen present in the small intestine after 2.5 hours is merely the difference between the nitrogen curve of the protein-free-fed rats and the protein curves at 2.5 hours (fig. 2) or approximately 2.7 mg per 5% increase in dietary protein; (2) fecal endogenous nitrogen excretion per gm of dry matter ingested is the same for the rats fed the protein or protein-free rations (fig. 3).

Thus, the figure substituted in the formula for "d" is obtained by subtracting differences at 2.5 hours from total nitrogen accumulation at 8 hours. The figure substituted in for "c" is obtained by multiplying the average amount of food consumed in 4 days by 1.52 (endogenous nitrogen secreted per unit of food consumed, fig. 3). Substitution of the data obtained with the 15% protein[8] ration into this formula gave a value of 1000 mg for the endogenous nitrogen secretions, slough-off, and the like, over a 4-day period. Thus, 125 mg of nitrogen might have been secreted per feeding with an adequate protein diet compared with the 130 mg of nitrogen ingested per feeding with the same diet. The assumptions made in carrying out this calculation might not be completely valid. The second assumption is especially open to question. For this reason, the derived value is meant only to be a rough approximation. The endogenous nitrogen obtained from this calculation, however, was in line with estimations of Rosenthal and Nasset.

Simple determinations of the gross intestinal nitrogen content do not suffice in studies of the magnitude of endogenous nitrogen secretions. Some index of measurement must be used and certain assumptions have to be made to estimate the relative contribution of endogenous nitrogen to the small intestinal nitrogen pool during the various time intervals of the experimental period.

The values calculated in this experiment are important because they demonstrate that a large turnover of nitrogen can occur within the digestive tract. The added importance of a large endogenous nitrogen pool in the intestine depends, to a certain extent, on the relative rates of absorption of endogenous and dietary nitrogen.

SUMMARY

Gastrointestinal contents of rats fed chromic oxide as an indicator and varying levels of whole-egg protein were analyzed at different time intervals after feeding for dry matter, nitrogen, and chromic oxide. Peak increases in the nitrogen content of the small intestine occurred at one and 8 hours after feeding. The nitrogen peak accumulation reached at one hour seemed to be of dietary origin. Accumulations of endogenous nitrogen secretions were principally responsible for the 8-hour peak increase. Endogenous nitrogen secretions appeared to increase with the level of protein in the diet. A method was proposed for the calculation of the magnitude of the endogenous secretions using nitrogen-chromic oxide ratios. Use of this method gave a value of 563 ± 147 mg for the nitrogenous secretions, slough-off, and the like, by the digestive tract over a period of 4 days (8 feedings) when feeding a protein-free ration. This figure was equivalent to the amount of nitrogen consumed in 8 feedings with a 10% protein ration.

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[8] Data from rats fed 20% of protein were not used due to the different nitrogen accumulation pattern noted with this ration.

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Mixture of Endogenous and Exogenous Protein in the Alimentary Tract[1]

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Ingestion of any meal sets off a series of neural, hormonal, chemical and mechanical stimuli which excite salivary, gastric, hepatic, pancreatic and intestinal secretory cells to pour out enzyme and other proteins into the lumen of the digestive tract. The magnitude of these responses indicates a large and rapid turnover of protein in the glandular tissues of the digestive tract. The hydrolytic enzymes, other secreted proteins, and sloughed mucosa provide enough endogenous amino acids to the mixture in the gut lumen to obliterate the unique chemical characteristics of the amino acid mixture derivable solely from the ingested exogenous protein (Nasset et al., '55). A calculation made from the amino acid composition of 5 digestive enzymes suggested that ingested protein is mixed in the gut lumen with at least an equal mass of endogenous protein (Nasset, '57). The feeding of test meals containing C^{14}-labeled casein, as described in the present paper, provides a more direct approach to this problem. The results indicate that exogenous protein in the small intestine is mixed with more endogenous protein than was previously suggested. Some results of the present work appeared in a preliminary report.[2]

METHODS

Two dogs were provided with jejunostomies about 50 cm caudad from the ligament of Treitz. They had been used previously for other experiments and were well trained. After 18 hours of fasting the test meal, containing 10 gm each of lard, cornstarch and radioactive casein,[3] was promptly ingested. Contents of the jejunum were aspirated through a catheter fitted with a terminal balloon that occluded the lumen distal to the stoma and helped divert contents to the outside. Pressure in the collection system was maintained at -11 ± 0.5 cm of Hg. The receiving flask was immersed in a salt-ice bath. The 4 samples, collected in 2.5-hour intervals, were frozen and dried in the frozen state. Further details are given elsewhere (Schreiber and Nasset, '59).

Three groups of rats were fasted two days before being given the test meal by stomach tube. The test meal contained (in per cent) C^{14}-labeled casein, 25; sucrose, 46; cottonseed oil, 17; salts, 4; cellulose flour, 6; and vitamin mixture in sucrose, 2. At 1, 2 and 4 hours, *post cibum*, animals were anesthetized with ether and the heart incised to cause exsanguination into the thorax. Ligatures were placed at cardia, pylorus and ileo-cecal junction and the stomach and small intestine quickly cut away from the mesentery. No blood was visible but the organs were rinsed three times in Ringer solution, dried on paper toweling and slit longitudinally. Gastric and gut contents were collected quantitatively in separate containers and the opened organs agitated in 5 successive portions (about 8 ml each) of Ringer solution. No visible extraneous material was left adherent to the mucosa after this treat-

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[1] Aided by a grant from the National Institutes of Health.

[2] Nasset, E. S. 1958 Fate of radioactive casein in digestive tract. Federation Proc., *17*: 486 (abstract).

[3] We greatly appreciate receipt of two lots of radioactive casein (18 and 29 μc/gm atom of carbon) which were gifts from Professor Max Kleiber, University of California at Davis. The casein was obtained from cows given C^{14}-labeled compounds intravenously. Details of its production, separation and purification are given by Black, Kleiber and Smith, J. Biol. Chem., *197*: 365, 1952.

ment and no trace of blood was visible. The washings were combined, dried in the frozen state and portions of the dried material analyzed. Total N was determined by the microKjeldahl method (Miller and Houghton, '45). The carbon in samples of test meal and gastrointestinal contents was converted quantitatively by wet combustion (Van Slyke et al., '51) to CO_2 which was then assayed for radioactivity with an ionization chamber and a vibrating reed electrometer.[4] It was assumed that the casein in the lumen, or the amino acid mixture derived from it, retained approximately its original C/N ratio. It is known that the absorption of single amino acids from isolated intestinal loops may vary considerably. In the normal process of absorption, however, a steady state seems to be established in which the whole assortment of amino acids disappears from the lumen in a rather uniform manner. If a few amino acids were absorbed at much greater rates than the others the digestion mixture should gradually become depleted of the more rapidly absorbed ones. There is no evidence to show that this happens in normal digestion and absorption (Nasset, '57). The possibility that the labeled amino acids in the radioactive casein were selectively held back or more rapidly absorbed seems rather remote. In one series, free amino acids were determined in 1% picric acid extracts (Tallan et al., '54) of dried intestinal contents of one rat at each of the 1- and 2-hour intervals and of both rats at 4 hours. Analyses were made by means of resin column chromatography with an automatic instrument similar to the one described by Spackman et al. ('58).

RESULTS

Results of experiments with two dogs are summarized in table 1. The volume of contents recovered from the jejunostomies in the two dogs varied appreciably in the 4 collection periods. The total volume obtained from dog B was only 59% of that from dog A. Total solids were nearly the same in both and total N recovered from dog B was 88% as great as from dog A. The total recovered exogenous N, attributable to the labeled casein, was 6.3% from dog A and 5.9% from dog B. Ignoring, as a first approximation, the possibility of significant recycling of the labeled material, it appears that each dog in 10 hours digested and absorbed approximately 94% of the ingested casein.

[4] We are indebted to Dr. Leon L. Miller of the Department of Radiation Biology for these determinations.

TABLE 1

Endogenous and exogenous N in intestinal contents collected from jejunostomies after feeding test meals containing C^{14}-labeled casein[1]

	Sample[2]				
	1	2	3	4	Totals
	Dog A				
Volume recovered, ml	47	40	154	133	374
Total solids recovered, gm	3.661	2.771	8.659	3.976	19.066
Total N, mg	162.5	117.2	249.4	258.0	787.1
Exogenous N,[3] mg	41.7	23.4	15.1	4.2	84.4
Endogenous N,[4] mg	121.8	93.8	234.3	253.8	702.7
Exogenous N, % of total	25.6	19.9	6.1	1.6	10.7
	Dog B				
Volume recovered, ml	24	71	38	88	221
Total solids recovered, gm	1.235	5.272	3.142	10.115	19.764
Total N, mg	66.5	260.4	135.7	232.6	695.2
Exogenous N,[3] mg	7.1	41.7	18.6	12.3	79.7
Endogenous N,[4] mg	59.4	218.7	117.1	220.3	615.5
Exogenous N, % of total	10.7	16.0	13.7	5.2	11.5

[1] Thirty grams of diet were fed containing 10 gm of radioactive casein and 1.350 gm of N.
[2] Samples 1, 2, 3 and 4 were collected from zero to 2.5, 2.5 to 5.0, 5.0 to 7.5, and 7.5 to 10.0 hours after feeding, respectively.
[3] Attributable to labeled casein.
[4] By difference.

In a preliminary series 6 rats were fed test meals containing 1.5 gm of labeled casein. At 1, 2 and 4 hours after feeding, two rats were sacrificed and the combined contents of stomach and small intestine were analyzed. Determinations of total N and radioactivity indicated that labeled casein accounted for 82% of total protein in the first hour, 76% in the second and 65% in the fourth. This indicates a gradual dilution of exogenous protein with endogenous protein but fails to indicate the relative contributions of stomach and gut.

The second series was fed test meals that contained 1.0 gm of labeled casein. The contents of stomach and gut were collected and analyzed separately. Results of these experiments are summarized in table 2. As expected, the N contained in the stomach was predominently exogenous. In the gut there was a striking reversal and only about 1/7th of the nitrogen was exogenous.

In a third series the test meal contained only 0.5 gm of labeled casein. The intestinal contents were analyzed for total N, radioactivity, and free amino acids (4 samples) at 1, 2 and 4 hours *post cibum*. Exogenous N constituted 7.4, 10.8 and 13.1% of total N in gut contents at 1, 2 and 4 hours. Amino acid analyses are expressed as relative molar ratios in table 3. The amino acid compositions of the gut samples resemble each other much more closely than the casein in the test meal.

DISCUSSION

The point of primary interest is the great dilution of exogenous N with endogenous N in the small intestine. In dog A it was 4-, 5-, 17-, and 61-fold and in dog B it was 9-, 6-, 7-, and 19-fold in periods 1, 2, 3, and 4 respectively. In the rat the dilution was 6- or 7-fold. Previous work and certain calcalculations (Nasset, '57) had led us to expect at least a two-fold dilution but scarcely as much as is here indicated. These results demonstrate that ingested protein is mixed with enough endogenous protein to mask the chemical identity of the ingested protein. This was first demonstrated when molar ratios of amino acids in gut contents were shown to be essentially independent of the type of test meal ingested (Nasset et al., '53). Total N is rapidly and extensively lost from pancreatic and intestinal tissue during starvation and very rapidly regained by the gut during realimentation (Ju and Nasset, '59). LeBlond and Walker ('56) estimated that an adult man may slough daily over 200 gm of digestive tract

TABLE 2

Endogenous and exogenous N in stomach and small intestine of rats after feeding a test meal containing C^{14}-labeled casein[1] (two rats used at each time interval)[2]

Time after feeding	Stomach	Intestine	Totals
1 Hour			
Total N, mg	129.8	25.9	155.7
Exogenous N,[3] mg	118.5	3.5	122.0
Endogenous N,[4] mg	11.3	22.4	33.7
Exogenous N, % of total	91.3	13.4	78.4
2 Hours			
Total N, mg	93.4	19.6	113.0
Exogenous N,[3] mg	88.5	2.8	91.3
Endogenous N,[4] mg	4.9	16.8	21.7
Exogenous N, % of total	94.7	14.3	80.8
4 Hours			
Total N, mg	51.2	19.5	70.7
Exogenous N,[3] mg	48.7	2.9	51.6
Endogenous N,[4] mg	2.5	16.6	19.1
Exogenous N, % of total	95.2	14.9	73.0

[1] Four grams of diet containing 1 gm of radioactive casein and 138.2 mg of N was fed by stomach tube.

[2] Only one gut sample was obtained at one hour.

[3] Attributable to labeled casein.

[4] By difference.

TABLE 3

Relative molar ratios (threonine = 1.00) of free amino acids in casein hydrolysate and small gut contents after feeding casein

Amino acid	Casein hydrolyzate[1]	Gut sample no.[2]			
		11	22	41	42
Alanine	1.01	3.11	3.58	3.37	2.98
Arginine	0.75	1.53	1.89	2.13	2.04
Aspartic	1.61	1.90	2.13	1.13	1.13
Glutamic	4.58	5.03	8.01	3.20	3.35
Glycine	0.68	2.65	2.79	1.99	2.29
Histidine	0.63	0.68	0.14	0.23	0.11
Isoleucine	1.57	1.25	1.00	1.11	1.10
Leucine	3.11	3.29	2.72	2.82	2.58
Lysine	1.59	2.50	2.08	1.83	1.86
Methionine	0.79	0.81	0.52	0.58	0.59
Phenylalanine	0.97	1.46	1.57	1.24	1.30
Proline	2.96	1.18	0.86	0.82	1.32
Serine	1.73	2.60	2.54	2.54	2.47
Threonine	1.00	1.00	1.00	1.00	1.00
Tyrosine	1.20	1.50	2.07	1.17	1.48
Valine	1.68	1.20	1.27	0.92	1.41

[1] Data for average casein in Amino Acid Handbook 1956 Block, R. J., and K. W. Weiss. Charles C Thomas, Springfield, Illinois, p. 266.

[2] Samples 11, 22, 41 and 42 taken at 1, 2, 4 and 4 hours after feeding respectively.

mucosa, which makes this another significant source of endogenous protein in the gut lumen. (Jejunal histological sections made in this investigation indicated that the mucosa remained intact during the experiment procedure). All of these endogenous proteins from whatever source seem to be hydrolyzed and very nearly completely recovered. Fecal protein apparently represents only a small fraction of the total protein turned over in the upper portion of the digestive tract.

Even when exogenous N in the gut has been reduced to 5% or less (period 4), presumably by digestion and absorption, the amount of endogenous N is still great (table 1). Are the nitrogenous compounds of endogenous origin incapable of stimulating the digestive organs to continued activity? Does the signal to cease secretory operations come from an empty stomach or some other portion of the digestive tract orad from the jejunostomy?

The experimental results obtained from dogs A and B may be considered temporal summations of the digestive processes at the point of the jejunostomy. The experimental results obtained from the rats may be considered spatial summations over the whole length of the stomach and the small intestine at three time intervals. Both sets of results point unmistakably to the conclusion that ingesta are mixed with relatively large quantities of endogenous materials, especially in the small intestine.

Earlier work (Nasset et al., '55) demonstrated that the molar ratios of amino acids in jejunal contents remained relatively constant whether the test meal contained egg albumin, zein or only carbohydrate and fat. The present results indicate a great preponderance of endogenous protein in gut contents and explain why ingested protein does not greatly influence the molar ratios of free amino acids in gut contents. The results in table 3 demonstrate again that the amino acid molar ratios in gut contents are largely independent of the molar ratios in the protein of the test meal. The ratios in gut samples resemble each other much more closely than the ratios of the casein which was fed. Alanine, arginine, glycine, lysine and serine show the differences in a striking manner. The two 4-hour samples (41, 42) are generally in good agreement. This close regulation of the amino acid mixture in the lumen of the gut probably exerts an important immediate influence on the protein metabolism of the intestinal mucosa itself and on the liver. The intestinal mucosa has first access to absorbed amino acids and some evidence indicates that it may remove its quota immediately from

the amino acid stream as it passes through the mucosal cells during the process of absorption (Ju and Nasset, '59). This subtraction, plus the intramural transaminations involving glutamic and aspartic acids (Neame and Wiseman, '57), could alter the mixture that appears in the mesenteric and portal veins and therefore the mixture available to the liver. Other work has shown that ingested protein does not affect the amino acid composition of portal blood in a predictable manner (Denton and Elvehjem, '54; Dent and Schilling, '49).

Some investigators have found it difficult to accept the fact of the presence of relatively large amounts of endogenous protein in the digestive tract during digestion (Geiger et al., '58) or the possibility that it may have nutritional significance (Rogers et al., '60). Our results in no way challenge the concept of the essentiality of certain amino acids or any of the other classical and well established fundamental concepts of nutrition. We wish to draw attention again to the fact that ingesting a meal elicits the responses described and that these responses will eventually be properly fitted into the overall mechanisms that govern ingestion and hence nutrition. These were acute experiments, involving only a single meal, and no conditioning or long-term after effects are concerned. An animal soon refuses food that contains an incomplete protein or amino acid mixture as the sole source of dietary N. Observation of the amino acid composition of gut contents under such conditions would be interesting. Perhaps some amino acid ratios may change sufficiently under such conditions to elicit promptly, from the intestinal mucosa or other tissue, the signals that impel the animal to stop eating an incomplete protein or amino acid mixture.

SUMMARY

Two dogs provided with jejunostomies were fed test meals containing radioactive casein as the sole source of protein. The jejunal contents were collected in 4 fractions over a period of 10 hours. The N attributable to labeled casein was diluted more than four-fold with endogenous N. Six rats were fed test meals containing radioactive casein by stomach tube. At 1, 2 and 4 hours after feeding, two rats were sacrificed and the gastric and intestinal contents analyzed separately. Exogenous N was diluted 6- or 7-fold with endogenous N and the molar ratios of free amino acids in gut contents were markedly different from those found in the ingested casein.

ACKNOWLEDGMENT

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The Biological Activities of 6-Methyl-7-chloro-9-(1'-D-ribityl)isoalloxazine and Dichlororiboflavin[1,2,3]

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A previous report (Haley and Lambooy, '60) presented a brief review of riboflavin antagonists possessing the isoalloxazine structure. It appeared that the more potent antagonists fell into two groups, analogs possessing foreign atoms in positions 6 or 7 or both and homologs possessing alterations in the substituents in positions 5, 6, 7 or 9. The available data for these several compounds indicate, however, that the most effective antagonists in the rat and in *Lactobacillus casei* were those which possessed a D-ribityl group in position 9 and various substituents in positions 6 or 7 or both.

Dichlororiboflavin (Kuhn et al., '43), the first and most widely cited antagonist of riboflavin, has been almost always referred to as a potent antagonist despite the fact that very little was known about the biological activity of this material. Kuhn and associates had shown that it was a reversible inhibitor of riboflavin in the nutrition of *Staphylococcus aureus* and *Streptobacterium plantarum*, neither of which require an exogenous source of riboflavin, and of *Bacillus lactis acidi* which does require the vitamin. Inhibition of the first two organisms was not sustained but was progressively released as the incubation time was lengthened. Ten years later, Snell and co-workers ('53) showed that dichlororiboflavin was essentially inert in the nutrition of *L. casei*.

As a result of what we had observed in our laboratory and the reports from other laboratories which were available at that time we had arrived at the opinion that the structure of the riboflavin molecule could not be altered extensively and still retain biological activity. This applied to both vitamin-like activity and inhibitor activity. We concluded that while there seemed little justification for classifying dichlororiboflavin as a potent inhibitor, it did show some inhibitory properties. We further considered that the structure of dichlororiboflavin was less like that of riboflavin than the structures of various homologs of the vitamin such as diethyl riboflavin (Lambooy and Aposhian, '52) and 6-ethyl-flavin (Lambooy and Aposhian, '60), two compounds which we had studied extensively. The activities of these two compounds varied from biological inertness to potent vitamin-like and potent inhibitor properties. A logical continuation of this line of thought led us to question whether we might not find compounds of potent inhibitor properties among some which could be considered as resembling dichlororiboflavin but which did not depart from the structure of riboflavin so extensively.

The two most likely structures to be investigated were 6-chloro-7-methyl- and 6-methyl-7-chloro-9-(1'-D-ribityl)isoalloxazine, compounds which represent changes of the methyl groups of riboflavin to chlorine atoms one at a time. We have synthesized these two compounds (Haley and Lambooy, '54) and have recently reported on the biological activity of one of them, the

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[2] Brief reference was made to a portion of this work at the Symposium on the Antimetabolites, Their Modes of Action and Therapeutic Implication, 10th Annual Meeting, The National Vitamin Foundation, Inc., New York, 1955.
[3] 6-Methyl-7-chloro-9-(1'-D-ribityl)isoalloxazine has been screened for anticancer activity by the Cancer Chemotherapy National Service Center.

6-chloro-7-methyl-flavin (Haley and Lambooy, '60). This compound was found to be the most potent inhibitor of riboflavin in the nutrition of the rat and of *L. casei* to have been described to that date. In both cases, however, the responses were unusual and did not follow the normal pattern of classical inhibition.

The 6-methyl-7-chloro-9-(1′-D-ribityl)-isoalloxazine is an even more potent antagonist of riboflavin in the nutrition of *L. casei* than the isomeric 6-chloro-7-methyl-flavin. Careful study has led us to conclude that in addition to being a mild inhibitor of riboflavin in the rat, it possesses very slight growth promoting properties.

The inhibitory properties of this new flavin for *L. casei* and its relatively harmless effects in the rat prompted us to investigate its potency as an inhibitor for several micro-organisms which do not require an external source of riboflavin but which are of clinical interest or importance. The flavin was found to be without influence on the growth of *Candida albicans, Hemophilus pertussia, Mycobacterium tuberculosis, Bacillus subtilis, Aerobacter aerogenes, Staphylococcus aureus, Salmonella paratyphosa* A and *Escherichia coli*. The isomeric 6-chloro-7-methyl-flavin was also found to be without influence on the growth of these micro-organisms.

Consideration of our findings relative to these two flavins led us to undertake a study of the biological activity of dichlororiboflavin. It was important for us to know the details of its activity in the rat so that appropriate comparisons might be made of these three compounds of related structures. Ohguri ('58) had reported on the use of dichlororiboflavin in the rat but no useful information was given relative to its influence on growth or survival.

We have prepared dichlororiboflavin by methods which are a considerable improvement over those used before, have investigated its activity in *L. casei* for confirmatory purposes only and have studied its activity for the rat in considerable detail. Ironically, dichlororiboflavin is completely inert as an antagonist of riboflavin in these two forms.

METHODS

Acid production by L. casei. The quantity of lactic acid produced by *L. casei* (ATCC 7469) was measured by titration with 0.1 N sodium hydroxide. The observed values were plotted against the flavin concentrations. One set of duplicate tubes (standard curve) was prepared by the routine procedure using graded increments of riboflavin (USP Reference Standard) from zero to 0.3 μg per tube. Another set of duplicate tubes (inhibition curve) was prepared to contain in all cases 0.3 μg

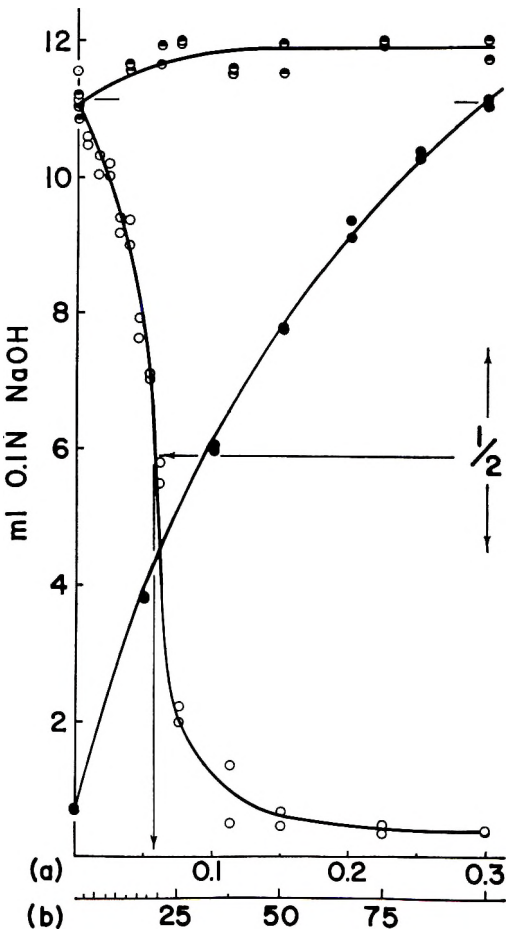

Fig. 1 Lactic acid production by *L. casei* in riboflavin (—●—), in mixtures of riboflavin and 6-methyl-7-chloro-flavin (—○—) and in mixtures of riboflavin and dichlororiboflavin (—◓—). (a) Riboflavin in μg per 10 ml of culture medium; (b) 6-chloro-7-methyl-flavin or dichlororiboflavin in μg per 10 ml of culture medium; each tube contained 0.3 μg of riboflavin.

of riboflavin per tube plus graded increments of the appropriate flavin from zero to 100 μg per tube. For the 6-methyl-7-chloro-flavin the tubes were prepared by adding to each the appropriate amounts of the flavin solutions and water to make a volume of 5 ml. After the tubes had been autoclaved, 5 ml of autoclaved basal medium were added aseptically and all were inoculated and incubated. This procedure was necessary because the analog was reversibly reduced when autoclaved in the presence of the basal medium. The remainder of the procedure and the determination of the inhibition index was like that outlined before (Haley and Lambooy, '60). The results are summarized in figure 1.

Rat growth. All studies with these materials were patterned after our procedure for the study of the 6-chloro-7-methyl-flavin. Male rats of the Wistar strain were used throughout. The conditions under which the animals were maintained and the riboflavin-deficient diet used were essentially the same as those described before. All flavin supplements were given by stomach tube as a solution or suspension in 0.5 ml of 6% gum acacia solution, each day for 28 days, immediately before the animals were fed.

The experimental procedures were begun on the several groups of animals after they were maintained for varying periods of time with a riboflavin-deficient diet. For the 6-methyl-7-chloro-flavin some animals were fed the deficient diet for two weeks before the test periods were begun. Other animals used for the study of this flavin were fed the deficient diet until they failed to show a net gain for a period of 14 days. The latter procedure was used throughout the studies with the dichlororiboflavin. The animal groupings and the supplements are adequately described in the appropriate tables, 1 and 2. The tabulated information by no means covers the extent to which the 6-methyl-7-chloro-flavin was studied but it contains all the significant information.

Other micro-organisms. Standard bacteriological procedures were used to study the responses of the micro-organisms listed in the introductory portion of this report. For several, the appropriate special me-

TABLE 1

Growth and survival of riboflavin-deficient rats given 6-methyl-7-chloro-flavin

Group no.	Supplement[1]	No. of animals/ No. of survivors	Av. weight gain of survivors ± S.E.[2]
			gm
1	None (deficient)	15/9	10±3
2	25 μgEq[3] A	10/4[4]	2±2
3	50μgEq A	9/9	12±4
4	500 μg A	17/13	26±4
5	1 mg A	10/7	39±6
6	20 μg Rb	14/13	96±3
7	20 μg Rb + 500 μg A	14/13	65±5

Probability (P) relationship between groups

	1	2	3	4	5	6	7
1	—	0.12	0.70	0.009	0.0003	"0"[5]	"0"
2	—	—	0.14	0.007	0.001	"0"	"0"
3	—	—	—	0.03	0.002	"0"	"0"
4	—	—	—	—	0.08	"0"	"0"
5	—	—	—	—	—	"0"	0.003
6	—	—	—	—	—	—	"0"

[1] A indicates analog, 6-methyl-7-chloro-flavin; Rb, riboflavin.

[2] Standard error.

[3] Micrograms equivalent, see footnote 6 in text.

[4] Some deaths were due to stomach tubing accidents, some were not. Average starting weights of animals in the groups, as a result of randomized distribution, were between 44 and 52 gm.

[5] P value less than 0.00001 (t values varying from 6 to 21).

TABLE 2

Growth and survival of riboflavin-deficient rats given dichlororiboflavin

Group no.	Supplement[1]	No. of animals/ No. of survivors	Av. weight gain of survivors ± S.E.
			gm
1	None (deficient)	8/8	16±3
2	50 μgEq DC	11/11	10±4
3	500 μg DC	8/7	11±6
4	2 mg DC	8/8	20±3
5	20 μg Rb	5/5	104±4
6	20 μg Rb+500 μg DC	5/5	102±3

Probability (P) relationship between groups

	1	2	3	4	5	6
1	—	0.28	0.43	0.34	"0"	"0"
2	—	—	0.89	0.09	"0"	"0"
3	—	—	—	0.18	"0"	"0"
4	—	—	—	—	"0"	"0"
5	—	—	—	—	—	0.70

[1] DC indicates dichlororiboflavin; Rb, riboflavin.

dium was employed with and without the addition of 500 μg of analog per plate (the first three micro-organisms listed). The remainder of the micro-organisms were tested at levels of zero, 1, 3, 10, 30 and 100 μg of either analog per ml of medium. Only 6-chloro-7-methyl-flavin and 6-methyl-7-chloro-flavin were studied.

Preparation of 6,7-dichloro-9-(1'-D-ribityl)-isoalloxazine. 1,2-Dichloro-4,5-dinitrobenzene (Kuhn et al., '43), 5.2 gm (0.022 mole), 6.0 gm of D-ribamine and 80 ml of 80% ethyl alcohol were heated under reflux for 5 hours. The reaction mixture was placed in the refrigerator until crystallization was complete. The crystals were collected and recrystallized twice from approximately 100 ml of 80% ethyl alcohol, the first time, crystallization being carried out at refrigerator temperatures and the last time at room temperature. The yield of 1,2-dichloro-4-nitro-5-D-ribityl-aminobenzene was 3.7 gm (50% of the theoretical amount) of orange crystals melting at 187 to 188°C, (calibrated thermometer).

The above ribitylaminobenzene, 2.56 gm, 0.1 gm of platinum oxide, 44 ml of glacial acetic acid and 9 ml of water were hydrogenated at 60 p.s.i. for three hours. The colorless solution was filtered from the catalyst directly into a solution of 118 ml of hot glacial acetic acid containing 1.46 gm of alloxan and 2.9 gm of boric acid. The solution was heated to its boiling point and then placed in the dark for two days. The solvent was removed at reduced pressure while on a steam bath, and the following materials were added to the contents of the flask, in order, each addition being removed by distillation as above before the next addition was made: 100 ml of absolute methanol (4 times in succession), 100 ml of water, 100 ml of absolute methanol. Finally 50 ml of water were added and the product cooled in the refrigerator. The crude flavin was collected on a filter and when dry it was dissolved in 40 ml of concentrated hydrochloric acid, decolorizing charcoal was added and the mixture filtered. To the hot filtrate 200 ml of hot water were added and the product allowed to crystallize; it was then refrigerated. A second recrystallization yielded 2.17 gm (69% of the theoretical amount) of dichlororiboflavin as bright orange needles with a decomposition point of 285 to 287°C[4] (dec.) (uncorrected).

RESULTS

Acid production by L. casei. In figure 1 is shown the acid production by the organism in response to graded increments of riboflavin and to mixtures of riboflavin

[4] Kuhn et al. ('43) reported the melting point of the ribityl-aminobenzene to be 183 to 185°C and that for dichlororiboflavin as 273 to 275°C (dec.).

and graded increments of the analogs. The ratio of the mixture of 6-methyl-7-chloro-flavin which supported 50% of the acid production by the same quantity of riboflavin alone can be determined from the graph as 18.8 μg/0.3 μg. The use of this ratio for the calculation of the inhibition index gives a value of 59.[5] Dichloro-riboflavin is essentially inert in the nutrition of *L. casei* throughout the range of concentrations used.

Rat growth. The results of the animal studies are so clearly shown in tables 1 and 2 that little comment is required. At 25 and 50 μgEq[6] per day 6-methyl-7-chloro-flavin (table 1) does not stimulate growth of the riboflavin-deficient rat. When the quantity of this analog is increased to 0.5 or 1 mg per day it has growth promoting properties. A reasonable estimate would equate the activity of 1 mg of this flavin with approximately 5 μg of riboflavin; about 0.5% of the activity of riboflavin, for the stimulation of growth of the riboflavin-deficient rat under the conditions of these tests. Dichlororiboflavin is inert.

Molar extinction coefficients. Molar extinction coefficients were determined for the 6-chloro-7-methyl-flavin and the 6-methyl-7-chloro-flavin. The compounds were dissolved in water at a concentration of 5.00 mg per liter and the optical densities at various wavelengths measured by a Beckman Model DU Spectrophotometer.

Riboflavin:
Maxima: 267 mμ, 33200; 373 mμ, 10650; 445 mμ, 12250
Minima: 304 mμ, 1200; 399 mμ, 6900

6-Chloro-7-methyl-9-(1′-D-ribityl) isoalloxazine:
Maxima: 269 mμ, 37200; 360 mμ, 8970; 446 mμ, 12600
Minima: 303 mμ, 1870; 389 mμ, 4500

6-Methyl-7-chloro-9-(1′-D-ribityl) isoalloxazine:
Maxima: 267 mμ, 31500; 366 mμ, 9650; 444 mμ, 12350
Minima: 302 mμ, 1700; 395 mμ, 6050

DISCUSSION

6-Methyl-7-chloro-9-(1′-D-ribityl)isoalloxazine is the most potent inhibitor of riboflavin in the nutrition of *L. casei* to be described to date. In view of the reported biological activity of 6-chloro-7-methyl-flavin, it seemed reasonable to expect that the 6-methyl-7-chloro-flavin might also be a potent inhibitor in the nutrition of the rat. It was found to be a very mild inhibitor in this animal and what activity it had in this respect was somewhat unusual. Only after extensive studies have we concluded that this flavin stimulates the growth of the riboflavin-deficient rat to a small degree. In this respect it is like diethyl riboflavin and 6-chloro-7-methyl-flavin. The latter two flavins kill the animals to which they are fed, whereas this new analog of riboflavin appears to be harmless to the rat when used as the sole flavin in its diet at 2 mg per day for 28 days.

Our interest in chemotherapy had led us to hope that we might find a compound whose structure and properties were sufficiently like riboflavin so that the less critical metabolic systems of micro-organisms could be inhibited by it. A corollary hope was that among such inhibitors we might find one whose structure and properties were, at the same time, sufficiently unlike riboflavin so that the critical metabolic systems of mammals would exclude it altogether. 6-Methyl-7-chloro-flavin appeared to satisfy these conditions, yet the growth of several micro-organisms which were able to make riboflavin to meet their requirements was uninhibited by this substance.

Kuhn and associates reported that the E_o' of dichlororiboflavin (at pH7) was −0.095 volts, while under the same conditions the E_o' for riboflavin was −0.185 volts. They state that, "dichlororiboflavin in its dihydro- or reduced form is a considerably weaker reductant than dihydroriboflavin." They further state that, "perhaps the dichlororiboflavin can not functionally replace riboflavin in the cell because the reduced form of the dichloro-

[5] The reference, Haley and Lambooy ('54), gave a value (I.I. = 22) which had been determined from a pH plot of acid production, a less precise procedure, and a value (I.I. = 28) which had been determined by a titratimetric procedure on a small number of culture tubes.

[6] Microgram equivalent, μgEq: a correction for the molecular weights of the two flavins amounting to 1.054 times the corresponding micrograms of riboflavin. For example 25 μgEq of analog is equal to 26.35 μg.

enzyme is not sufficiently negative to reduce oxygen to hydrogen peroxide." This is another way of stating what they believe to be the reason that dichlororiboflavin does not possess vitamin-like action, and not, as has sometimes been cited, why they consider it to be an inhibitor. There is, however, no evidence that the dichlororiboflavin is reduced to the dihydro-form in the cell. The authors did not propose that the more positive E_o' of the dichlororiboflavin is the reason that it is an inhibitor nor did they propose that the dihydro-form of the compound is the inhibitor.

We are now able to present evidence that the more positive E_o' for dichlororiboflavin of itself may not be the reason that it is an inhibitor. It is not, for example, an inhibitor in *L. casei* nor does it inhibit the utilization of riboflavin in the rat. Might we assume that it is just enough too positive for the organisms which it does inhibit but that it is too much too positive to inhibit *L. casei*? This possibility is ruled out by the observation that 6-methyl-7-chloro-flavin, which has an E_o' which appears to be still more positive than that of dichlororiboflavin, is the most potent inhibitor of riboflavin in *L. casei* to have been described.

What evidence do we have that 6-methyl-7-chloro-flavin has a more positive E_o' (is a stronger oxidizing agent) than that possessed by dichlororiboflavin? It is the only flavin we have encountered which is reduced by the basal medium during autoclaving. This reduction is unusual in that the oxidation state of the flavin is poised at a "rhodoflavin" state (Michaelis et al., '36; and Kuhn and Strobele, '37) representing a partial reduction to a "leucoflavin." The product is a red nonfluorescent material which can be returned to its original oxidized state immediately by treatment with hydrogen peroxide or nitric acid or more slowly by aeration. Reduction to the "leucoflavin" form is accomplished immediately by the addition of sodium hydrosulfite. The presence of the added L (−) cystine in the basal medium is responsible for this conversion.

When *L. casei* was grown in the presence of a mixture of 6-chloro-7-methyl-flavin and riboflavin, a modified form of this organism emerges which is able to utilize the analog. A modified form of this sort did not appear in the case of the 6-methyl-7-chloro-flavin. The modified form of *L. casei* which responded quantitatively to the 6-chloro-7-methyl-flavin showed no response to the isomeric 6-methyl-7-chloro-flavin.

6-Chloro-7-methyl-flavin stimulated the growth of the riboflavin-deficient rat to the same extent as one-half the same quantity of riboflavin. Its inhibitory properties could be expressed only in terms of its highly lethal action. 6-Methyl-7-chloro-flavin has extremely low growth stimulating potency, yet its inhibitory properties can be observed in the effect it has on the utilization of riboflavin when the two are given simultaneously. The addition of 500 μg of the analog to a 20-μg supplement of riboflavin reduces the average weight increase from 96 to 65 gm (table 1). We found in other smaller groups of animals that the addition of 40 μg and also 200 μg of riboflavin to the supplement of 500 μg of the analog stimulated an average weight increase of 84 ± 4 and 128 ± 4 gm, respectively, during the same length of time. Increasing the amount of riboflavin in the presence of the same amount of the analog causes a better growth response. Is this the same as stating that increasing the amount of riboflavin under these circumstances causes a progressive decrease in the inhibitory action of the analog?

If 500 μg and 1 mg of the analog cause a significant growth response in a riboflavin-deficient rat and in so doing appears to be harmless for the animal, can this substance be called an inhibitor? It would be an inhibitor in the sense that it conceivably excluded the riboflavin molecules from the active enzyme sites which it occupied and thus reduced the utilization of the riboflavin. Whether the agent which interferes with the utilization is itself active or inactive as a growth stimulant is immaterial, such a substance is an inhibitor. It is an inhibitor for which an inhibition index is difficult to obtain if one uses growth as the criterion, for while a mixture of the two flavins might supposedly be independently competing for the same sites to accomplish a common

end, one functions with efficiency, the other with inefficiency.

Although 6-methyl-7-chloro-flavin stimulates the growth of the riboflavin-deficient rat, we do not claim for it vitamin-like properties. It simply can not qualify on the bases of structural, chemical and physical properties. We propose that it stimulates growth of the riboflavin-deficient rat by a mechanism which we believe to be characteristic of diethyl riboflavin and 6-chloro-7-methyl-flavin also, namely, the displacement of riboflavin from biochemically inactive and perhaps even active sites and thereby making at least part of this riboflavin available to sites which are active or which have a specific requirement for riboflavin.

The value of recording clear-cut differences which exist in the biological responses to compounds of close structural similarity justifies a summary of the action of these three closely related flavins. Both 6-chloro-7-methyl-flavin and 6-methyl-7-chloro-flavin are potent inhibitors of riboflavin in the nutrition of *L. casei*. Both compounds stimulate the growth of the riboflavin-deficient rat; one profoundly and the other only slightly. The mechanism by which this growth is accomplished is believed not to be due to unqualified vitamin-like activity; certainly not in the case of the very toxic 6-chloro-7-methyl-flavin nor of the apparently harmless 6-methyl-7-chloro-flavin. These two compounds are inhibitors of riboflavin in the nutrition of the riboflavin-deficient rat.

Although dichlororiboflavin is completely devoid of biological activity in the nutrition of *L. casei* or the rat, it is interesting that the 6,7-dichloro-sorbityl-flavin is also devoid of biological activity in *L. casei* and the rat and yet it causes the regression of established lymphosarcoma in the rat.

SUMMARY

6-Methyl-7-chloro-9-(1′-D-ribityl)isoalloxazine has been found to be a potent inhibitor of riboflavin in the nutrition of *Lactobacillus casei*. It did not, however, inhibit the growth or metabolism of several clinically interesting or important micro-organisms.

This new flavin has been found to stimulate a small growth response in the riboflavin-deficient rat. When administered simultaneously with dietary riboflavin it interferes with the utilization of the latter and is, by virtue of this action, an inhibitor, although a relatively weak one.

The observation that dichlororiboflavin is inert for *L. casei* has been confirmed and it has been found to be inert in the nutrition of the riboflavin-deficient rat as well.

ACKNOWLEDGMENT

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A Tubular Coprophagy-Preventing Cage for the Rat[1]

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During a study on the effect of irradiation on beef, male rats were observed to develop hypoprothrombinemia due to vitamin K deficiency, whereas the female rats were resistant to this deficiency of vitamin K (Metta et al., '59). For a study of vitamin K nutrition of the rodent it became necessary to eliminate coprophagy, since feces have been shown to be a source of vitamin K for the rat (Mameesh et al., '59; Barnes et al., '59). Barnes and coworkers ('57) have used a tail cup to prevent coprophagy in the rat. In our laboratory, however, this method has not proved ideal, particularly for the female rat. In this paper we shall describe a tubular coprophagy-preventing cage which the authors have designed for rats, and shall report some food intake, growth and coprophagy data which we have obtained using such cages.

EXPERIMENTAL

Tubular cages (fig. 1) were constructed of half-inch-mesh hardware cloth. Four pieces (one 18 inches × 5.5 inches and three 12 inches × 5.5 inches) were bent to form tubes with a diameter of 1.7 inch, and the adjacent edges were soldered. One of the ends of the short tube, both the ends of the other two short tubes, and one of the ends of the long tube were then cut at 45° angles. The tapered ends of the second and the third tubes were joined and soldered after trimming to avoid too much room at the corners. The tapered end of the long tube was then similarly soldered with the other end of the third tube, keeping all these three tubes in a horizontal plane. A hole with a 5.5-inch circumference was cut in the long tube 6.8 inches from its open end so that one end of the remaining short tube could be soldered around the hole and the other tapered end soldered to the tapered end of the other short tube. Another hole two inches in diameter was now cut into the bottom of the overhang, nearest its open end. A two-inch metal jar top (from which a circular portion was cut and the edges filed) was soldered around the two-inch hole at the bottom of the overhang. A feed jar could then be screwed into this top. A slit was cut ¼th inch from the open end of the overhang, into which an oval piece of metal could be slid to act as a door. In order to study rats for various lengths of time, graded sizes of tubes were constructed. The tubular cage was then secured on two raised horizontal rods, and a water bottle was adjusted at one corner of the tube away from the feed jar.

Rats placed in these cages can move along quite comfortably, but they cannot turn around to reach their excreted feces, which easily fall through the half-inch mesh. Rats housed in these tubular cages are easily weighed, since the cage is light and can be detached readily from its position on the rods and weighed with the rat inside the cage.

It may be argued that because of restricted activity the rat may consume less food than he would otherwise eat. Therefore suboptimal growth could be the result of decreased food intake and/or decreased recycling of feces which might contain some unknown nutritional factors not supplied in the diet. Two experiments were undertaken to study the effect of confinement in these coprophagy cages on food intake and growth.

Experiment 1. A synthetic diet, containing all known nutrients required for

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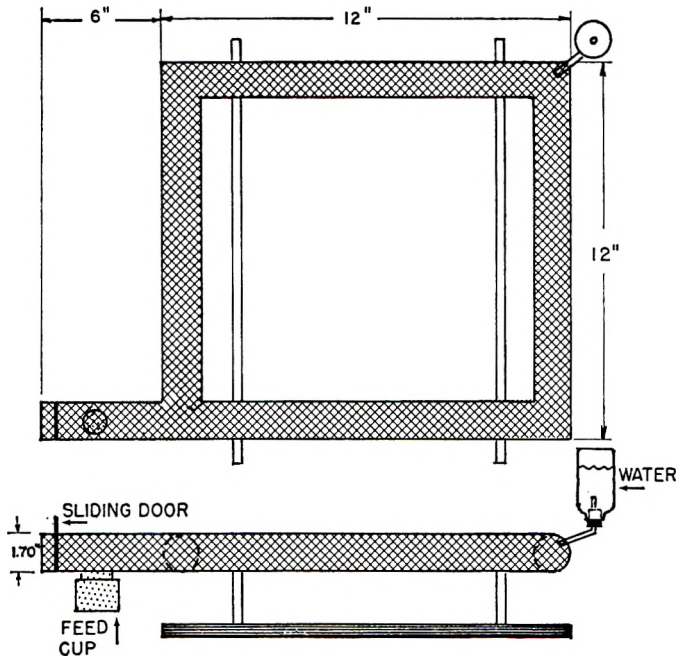

Fig. 1 A tubular coprophagy-preventing cage. The diameter of the cage will depend upon the size of the rat being studied.

TABLE 1

Performance of rats housed in coprophagy-preventing cages compared with that of rats wearing tail cups

Treatment	No. of rats	Food intake	Gain in body weight
		gm/day	*gm/day*
Experiment 1 (16-day test)			
Rats wearing tail cups	5	8.1	4.8
Rats housed in tubular cages	5	9.2	5.7
Experiment 2 (23-day test)			
Rats wearing tail cups fed ad libitum	5	same	4.2
Rats housed in tubular cages ⌊ pair-fed	5	same	4.2
Rats housed in regular cages ⌋ with group 1	5	same	4.7
		% of feces collected[1]	
Experiment 3 (19-day test)			
Rats wearing tail cups	4	43.3 ± 12.66[2]	
Rats housed in tubular cages	4	50.6 ± 3.46	

[1] $\frac{\text{Feces from tubular cages (gm)} - \text{feces from regular cages (gm)}}{\text{Feces from regular cages (gm)}} \times \frac{100}{1}$

[2] Standard error.

the rat including vitamins K and B_{12}, was fed ad libitum to 10 weanling rats for 16 days. Half of the rats were housed in coprophagy-prevention cages, and the other half were housed in regular cages and wore tail cups. Two sizes of coprophagy cages were used during the 16-day period.

Rats housed in the tubular cages ate more feed (9.2 vs. 8.1 gm/day) and gained weight more rapidly (5.7 vs. 4.89 gm/day) than the animals wearing tail cups (table 1). It appears that wearing tail cups has a greater effect on feed intake and body weight gain than does living in tubular cages.

Experiment 2. A second experiment was undertaken in order to test whether the slower gain in weight of rats wearing

tail cups in experiment 1 was the result of a decreased food intake or of the tail cups *per se*. The synthetic diet was fed for 23 days to 15 rats in 3 groups.

Group 1: Rats wearing tail cups, fed ad libitum

Group 2:
Rats housed in tubular cages } pair-fed with group 1
Group 3:
Rats housed in regular cages }

The animals with tail cups and those in the tubular cages gained weight at the rate of 4.2 gm per day (table 1). Therefore it would appear that the tail cups or tubular cages do not affect growth differently, as long as the feed intake is the same. Rats in the regular cages, however, gained 4.7 gm per day. These rats were apparently under less "stress" than were those wearing tail cups or living in tubular cages, or they were obtaining some extra "factor" by coprophagy.

Experiment 3. In this experiment we used both the tail cup and the tubular cage method to measure the amount of coprophagy practiced by rats. In order to do this we compared the amount of feces collected by each of these methods with the amount of feces collected from rats housed in regular cages. The comparison can then be expressed as a percentage.[3]

The test rat was moved into a tubular cage after it had received a constant amount of diet at the same time each day for two to three weeks. After a three-day acclimatization period it was assumed that all recycled feces had been utilized, and feces were collected for 24 hours from the time of feeding on the 4th day. The rat was returned to the regular raised cage on the 5th day, the same amount of food was offered immediately, and feces were collected for 24 hours. The feces were dried at 90°C for 6 hours and weighed.

We measured the amount of feces collected from rats wearing tail cups as compared with the amount collected from those rats housed in regular cages, and again from the same rats housed in tubular cages (table 1). The average percentage of coprophagy when the rats wore tail cups as compared with that when the rats were in regular cages was calculated as 43.4 ± 12.66%, whereas when calculated on the basis of the tubular cages versus the regular cages it was 50.6 ± 3.46%. The smaller variation in the values obtained when using tubular cages suggests that this method reduces the error previously encountered, whereas the lower value is an indication that a smaller amount of feces was collected from the tail cup than from under the tubular cage.

DISCUSSION

Radioactive chromium has been used to measure coprophagy in stanchioned rabbits (Thacker et al., '55), but stanchioning is not applicable to the rat. The tubular cages described in this paper are inexpensive, easy to construct and durable, and rats housed in these cages eat and grow well. Geyer et al. ('47) have constructed a circular type, as well as a short tubular type, of coprophagy-preventing cage for the rat. They have observed a maximum growth of 3.7 gm per day for rats housed in the tubular cages, and 4.5 gm per day for the rats in their circular cages, compared with 5.0 gm per day for rats housed in regular cages. Rats housed in our tubular cages and fed ad libitum grow at the rate of 5.7 gm per day.

The tail cup method has been satisfactory for the prevention of coprophagy in the male rat for short periods of time, but it appears to affect feed intake and gain in body weight adversely. In addition, it is unwieldy and impractical for the female rat, urination into the tail cup resulting in fermentation, with subsequent irritation and inflammation of the tail. As it is impossible for the rat in the tubular cage to turn around, and as the feces easily fall through the mesh, coprophagy is simply and effectively prevented in both male and female rats. The small variation in the measurement of coprophagy also reflects the efficiency of the tubular cage.

SUMMARY AND CONCLUSIONS

Tubular-type cages designed to prevent coprophagy in the rat are described. The cages are made with half-inch wire mesh

[3] $\frac{\text{Feces from tubular cage (gm)} - \text{feces from regular cage (gm)}}{\text{Feces from regular cage (gm)}} \times \frac{100}{1} = \frac{\%\ \text{coprophagy}}{(\text{or }\%\ \text{recycled})}$.

and are about 7 to 12 inches square, with an overhang for the feed cup. The rats seem to be comfortable in these cages, eat well and grow satisfactorily (5.7 gm/day).

The cages are completely effective in preventing coprophagy by both male and female rats and permit simple measurement of the amount of coprophagy practiced by rats housed in regular cages.

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Effects of Exercise and Reserpine Treatment on Heart Rate During Thiamine Deficiency in the Rat[1]

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The mechanism of thiamine deficiency bradycardia in rats remains obscure. The increased concentration of blood borne metabolites such as lactate, pyruvate and methyl glyoxal is insufficient to cause the slowing (Haynes and Weiss, '40).

Thiamine deficiency is associated with an accumulation of acetylcholine-like substances in the heart (Beznak, '56a). Vagotomy does not however result in any increase in heart rate but rather the reverse (Beznak, '56b). These findings suggest that the slowing may be the direct result of a reduced level of thiamine (Lowry, '52) and cocarboxylase (Wright and Scott, '54) within the heart, though experiments on isolated atria from thiamine-deficient animals do not bear out this interpretation (MacEachern and Brophy, '42).

This paper deals with an alternative possibility, namely, that the lowered heart rate reflects changes in the activity or capacity of the cardiac accelerator mechanisms.

Forced exercise is used to provide an index of cardiac acceleration capacity. Reserpine treatment is used to deplete cardiac catechol amines (Bertler et al., '56; Burn and Rand, '58; Muscholl, '59) and so knock out the sympathetic accelerator mechanism.

Estimates of "normal" heart rate vary widely between these extremes depending upon the way in which it is measured. Sometimes results are not consistent (Robertson and Doyle, '37). A consideration of the influence of measuring techniques on the heart rate is included in this paper. Conditions for measuring an arbitrary "resting" rate which gives consistent results are defined. With this method it is possible to detect thiamine deficiency in its early stages. Measurements were made on rats when the hearts slowed to a level of about 20% below the controls so that secondary, nonspecific effects which may occur when the heart rate falls to very low levels may be avoided.

EXPERIMENTAL

White male, Wistar strain albino rats of 50 to 100 gm were used. The technique for rendering the animals thiamine deficient and the method of pair feeding was the same as that previously described in papers from this laboratory (Beznak and van Alphen, '55).

Heart rate was either measured under anesthesia with needle electrodes or in unrestricted rats with chronically implanted electrodes. The implantation procedure was as follows: small incisions were made in the skin of the scalp, the back of the neck and on the back just anterior to the tail. A silver wire covered with polythene was threaded through a large needle. The needle was inserted into the scalp incision and pushed below the skin to the neck incision where the wire was brought out. Another wire was threaded below the skin from the tail incision to the neck. The bare ends of the wires were sewn in a loop beneath the scalp and the skin anterior to the tail and the skin sutured over. The neck wires were brought through the center tube of a stud-shaped plexiglass holder, the base of which was sewn beneath the skin. Two small tinned ends of wire were left projecting which could be easily soldered to fine leads in a few seconds.

After amplification the electrocardiogram (ECG) was fed into a tachometer similar to

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[1] This work was supported by a grant from the National Research Council of Canada.

that described by Roy ('59) for measuring human heart rates. The tachometer pulse duration was reduced to 80 msec. by substituting an appropriate C-R network and the time constant of the integrating circuit was reduced appropriately so that the high rate of the rat heart could be recorded. This instrument gives a linear response over the range of zero to 12 beats per second and it was calibrated to an accuracy of within 0.05 beats per second. The ECG was monitored continuously to insure that each R wave triggered one pulse in the tachometer. This was done most easily with a "standing" ECG using the R wave to trigger the fly back as previously described (Mainwood, '59). With appropriate filtering, the heart rate could be counted even during exercise since the electrodes did not lie over muscle masses. In preliminary experiments effects of handling, anesthetics and spontaneous activity on heart rate were measured. The standardized procedure finally adopted for measuring "resting" heart rate was to attach the leads and replace the animal in its cage. The animal was then watched until it settled down and spontaneous activity, apart from nose twitching, ceased for at least three minutes. This was found to give the most reproducible results.

The rats were exercised by putting them in a cage, the floor of which was an endless belt driven by a horizontal kymograph. Two parallel horizontal wires about half an inch from the floor at the rear end of the cage were connected to a condenser charged to 150 volts. The rats very soon learned to run when the motor was switched on. Reserpine was given intraperitoneally in a solution containing 0.2 mg per ml or 5 mg per ml of crystalline reserpine[2] in ascorbic acid. The effect of any kind of intraperitoneal injection in the unanesthetized rat was to cause some increase in resting rate for about 10 to 20 seconds. Apart from this, the vehicle had no effect. Dose levels of 5 mg per kg or sometimes 2 mg per kg were used. Where pentobarbital sodium[3] was used it was given in a solution containing 20 mg per ml at a dose level of 0.2 ml per 100 gm. Ether anesthetic was administered by holding an inverted beaker containing an ether soaked swab over the animal until it became quiet.

RESULTS

Effects of spontaneous activity, handling, ether and pentobarbital sodium. Some rats show considerable spontaneous activity particularly when placed in new surroundings. A fairly close correlation was observed between heart rate and the degree of activity. The results of one experiment are shown in figure 1. Heart rate was followed for 30 minutes and at the same time a second observer graded the activity level of the rat in arbitrary units. After 20 minutes the animal was put in the ether chamber. In this case activity caused a 30% fluctuation in rate, ether caused a 50% increase above the resting level. The activity response is typical but the response to ether is very variable and sometimes a fall in rate was observed even in the same animal. Handling the rats caused variable increases in rate which lasted up to 20 seconds after the rat had been replaced in the cage. Sudden noises had a similar effect.

In order to get reasonably reproducible results, rats were allowed to move in their cage under quiet conditions until they settled down and remained still, apart from nose twitching, for a period of at least three minutes. The rate meter needle was then watched until it gave a steady rate over a period of 10 seconds. This procedure was carried out on one group of 10 rats maintained with a thiamine-deficient diet and 7 controls fed a diet with thiamine supplement for the same period. The rates after supplying the diet for 4 weeks are shown in table 1 and are compared with measurements made on the same rats under ether. Although ether increased the mean rate difference by accelerating the normal rat hearts more than the deficient ones the difference was less significant because of the variability introduced by the ether.

Pentobarbital sodium was also found to have some accelerating effect on heart rate but its effect was much more uniform than that of ether. Thus one group of 12 normal rats showed a mean heart rate of 7.3 beats per second with a variance of 0.33 when measured under pentobarbital so-

[2] Serpasil, gift from Ciba Company Limited, Montreal.

[3] Nembutal, Abbott Laboratories.

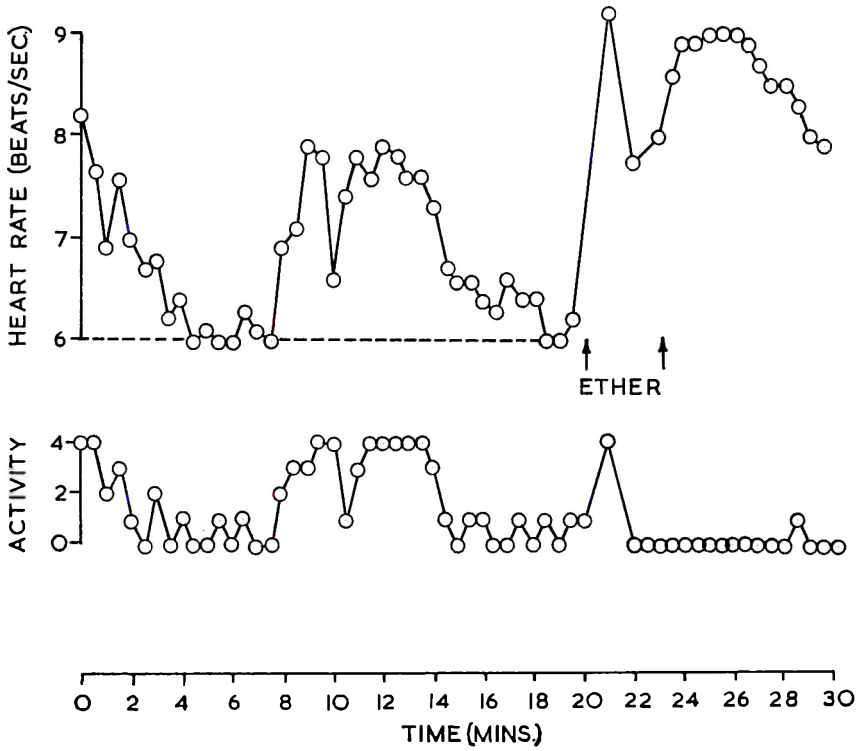

Fig. 1 Effects of spontaneous movement and ether anesthesia on heart rate in the rat. Activity is scaled in arbitrary units by the observer so that (1) indicates head movements; (2) head, neck and forelimb movements; (3) body movements or slow creeping around; (4) fairly rapid body movements or vigorous grooming; (5) is reserved for spontaneous running or climbing and forced exercise. During ether anesthesia the heart rate rises to a level seen only during forced exercise. The resting rate in this experimental run was taken to be 6.0 beats per second.

TABLE 1

Effect of ether anesthesia on heart rate in normal and thiamine-deficient rats

	Unanesthetized	Ether	P[1]
	Means		
Normal (7)	6.4	7.7	$0.05 > P > 0.02$
Vitamin B_1-deficient (10)	5.5	6.4	$0.05 > P > 0.02$
P	$0.01 > P > 0.001$	$0.05 > P > 0.02$	
	Variances[2]		
Normal (7)	0.07	1.79	< 0.001
Vitamin B_1-deficient (10)	0.31	1.09	0.05
P	0.05	—	

[1] Probability values based on "t" test between appropriate pairs in the case of mean values.
[2] Bartlett's test shows a significant inhomogeneity of variance ($0.01 > P > 0.001$).

dium at the same time 6 unanesthetized resting rats gave a rate of 6.3 beats per second with a variance of 0.25.

The effects of reserpine on heart rate. In either an unrestricted rat or a rat anesthetized by pentobarbital sodium, reserpine causes a slow decline in heart rate which falls to its lowest point after about 24 hours and then slowly returns to normal in 3 to 4 days. Vagotomy or atropine have no effect on this response. Parallel with this decline in rate is an increase in the response of the heart to intravenous injections of adrenaline or noradrenaline. For

instance the intravenous injection of 2.0 μg of adrenaline in one anesthetized, atropinized rat caused a barely detectable acceleration of 0.2 beats per second which lasted less than one minute. Three hours after a dose of reserpine the rate had fallen from 7.8 to 5.3 beats per second and it responded to 2.0 μg of adrenaline with an acceleration of 2.0 beats per second remaining elevated for over 10 minutes. Thus the acceleration was 10 times greater and lasted more than 10 times longer following reserpine treatment.

A dose of 2 mg per kg of reserpine is almost as effective in slowing the heart as a dose of 5 mg per kg. A second dose of reserpine 24 hours after the first is much less effective but usually caused a further slight slowing. The response of 12 thiamine-deficient and 12 normal rats to reserpine is shown in figure 2. All these values were obtained under pentobarbital sodium anesthesia. Evidently some factor, probably the repeated doses of the anesthetic, causes a small decline in rate in the control injected rats of both groups. The heart rate of the thiamine-deficient rats appears to be affected more than that of the normal rats. The final heart rate of the thiamine-deficient rats receiving two 5-mg doses of reserpine is only 69% of the control value whereas the normal rats have a heart rate of 80% of the control value.

Effects of exercise on heart rate. During exercise heart rate rises until it reaches a plateau after about two to three minutes. This rate is maintained, with small fluctuations, for the duration of the exercise. When exercise stops, the rate slowly declines to reach the pre-exercise level about 5 minutes later. The resting and exercise rates were measured in 6 normal and 6 thiamine-deficient rats. Both the resting and exercise rate were found to be lower in the thiamine-deficient rats though the mean increase during exercise was found to be identical in both groups (2.7 beats per second). The response to

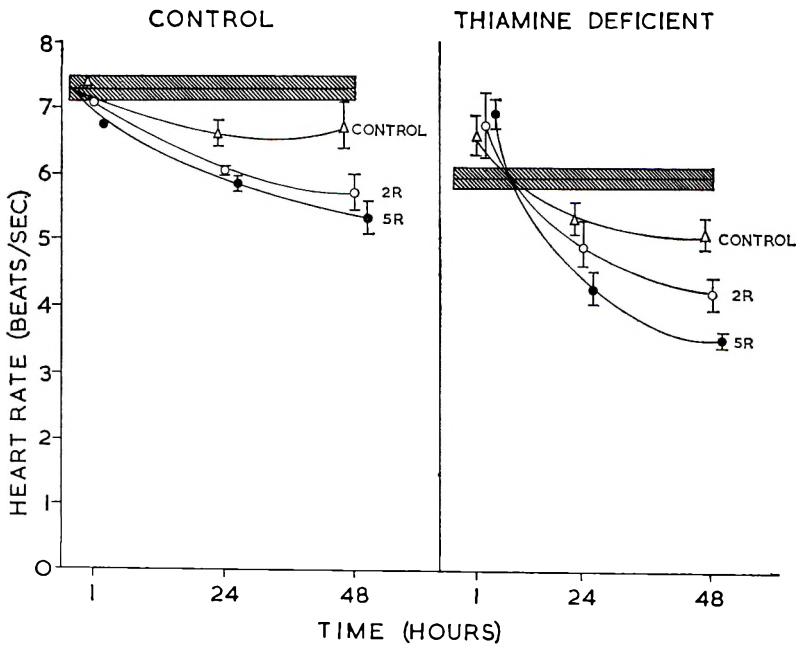

Fig. 2 Effect of reserpine on heart rate in rats measured under pentobarbital sodium anesthesia. The horizontal line bounded by the hatched area indicates the mean and standard error of the mean of 12 normal (left) and 12 thiamine-deficient (right) rats. Each group was divided into three groups of 4. At zero time intraperitoneal injections of ascorbic acid vehicle (△), reserpine at 2 mg per kg (○) and 5 mg per kg (●) were given. Heart rates were measured at one, 24 and 48 hours. After the 24-hour measurement injections were repeated. Horizontal bars on each side of the mean, mark off the standard error of the mean.

exercise was again measured in each of the groups 24 hours after an intraperitoneal injection of reserpine (5 mg per kg). The results are shown in figure 3. The reserpine injection is followed by a decrease in both the resting and exercise rates, although the decrease in exercise rate is considerably greater. The hearts of the thiamine-deficient rats show a greater response to reserpine in both the resting and exercise states.

Cardiac acceleration occurred in both groups during exercise despite the reserpine treatment. The response was not typical of that found in rats not receiving reserpine because of the abrupt onset of the acceleration and the very rapid decline after the exercise stopped when heart rate

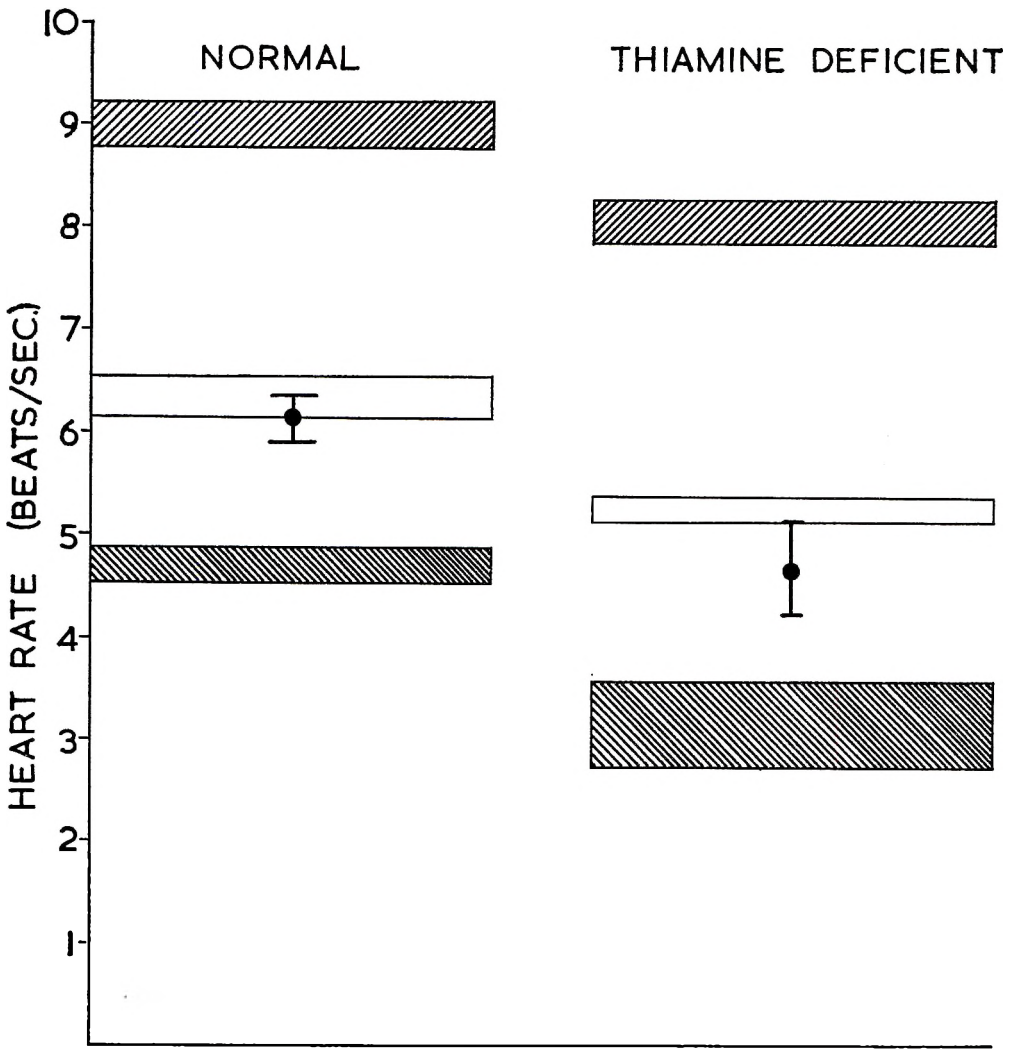

Fig. 3 The open horizontal strip indicates the resting heart rate bounded by one standard error on either side of the mean in 6 normal (left) and 6 thiamine-deficient (right) rats. The upper shaded areas indicate the mean and standard error during forced exercise. The lower shaded areas indicate the mean and standard error of the same rats 24 hours after an injection of reserpine (5 mg per kg) in the resting state. The black circle and horizontal bars indicate the mean and standard error of the mean of the reserpine treated rat during forced exercise.

sometimes fell below the pre-exercise level. The rate of recovery of heart rate was followed in one normal rat at intervals after the reserpine injection. Before treatment the time required for the heart rate to decrease half way to the pre-exercise level was 70 seconds. Three hours after reserpine administration the corresponding time was 13 seconds, and 24 hours later the heart rate had returned half way to normal in only 7 seconds.

The immediate effects of reserpine. Following a dose of reserpine there is little detectable change in the heart rate of normal unrestrained rats for about two hours, apart from the transient increase associated with handling the animal. Thiamine-deficient rats, conversely, showed an initial acceleration before the slow decline. During the first hour the heart rate of the thiamine-deficient rat increased well above that of the control normal animals. In one experiment three thiamine-deficient, three pair-fed and three normal animals were each injected with reserpine and the time course of the response was measured for three hours. The response of each of the rats to a second dose of reserpine was again measured 24 hours later. The results are shown in figure 4. In each of the thiamine-deficient rats there was a two-phasic acceleration with a first peak about 10 minutes after the injection and a second peak about one hour later. A small first peak was also noted in the pair-fed rats but prolonged acceleration was found only in the thiamine-deficient animals. The acceleration is most marked in unanesthetized rats though there is a small initial acceleration in the thiamine-deficient animals under pentobarbital sodium anesthesia (fig. 2).

DISCUSSION

Heart slowing during the early stages of thiamine deficiency is most clearly shown by measuring the rate in resting unrestrained rats in quiet conditions. This is because of the reproducibility of measurements and lower variability within groups compared with methods involving restraint by holding (Robertson and Doyle, '37) or light ether anesthesia (Beznak, and van Alphen, '55). Fairly reproducible results are also given if heart rate is measured a standard time after giving pentobarbital sodium. This is more convenient since it does not involve electrode implantation, though the mean heart rate in both thiamine-deficient and normal rats is higher than in the resting unrestrained rats.

An estimate of the capacity of the heart to accelerate was made in thiamine-deficient and normal rats. Both the normal and thiamine-deficient rats showed an almost identical acceleration (2.7 beats per second) above the resting level under standardized forced exercise, though the absolute rate was higher for the normal rats.

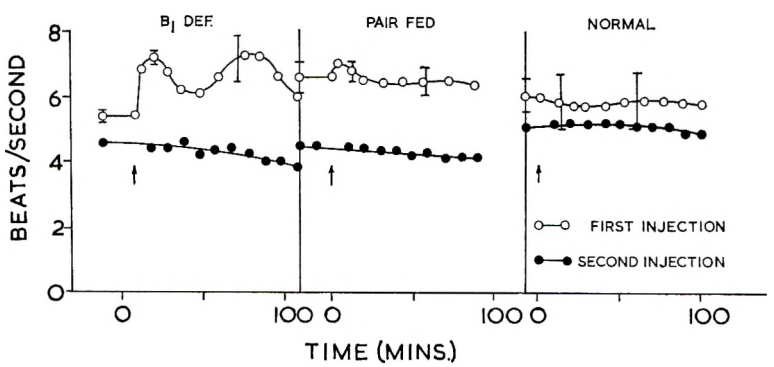

Fig. 4 The immediate response of heart rate to reserpine injections in unanesthetized normal, thiamine-deficient and pair-fed rats. Each group contains three rats, open circles indicate heart rate before and following the first injection of reserpine (5 mg per kg), the standard error of the measurements is indicated before the injection and at the peaks 10 and 60 minutes after the injection. The black circles indicate the response to a second injection of reserpine 24 hours later.

Reserpine lowers the heart rate in all groups of rats. It has least effect on resting unanesthetized normal rats, somewhat greater effects on rats anesthetized by pentobarbital sodium and a fairly large effect on exercising rats. The fall in rate is paralleled by a marked increase in the response of the rat heart to intravenous injections of adrenaline or noradrenaline, which normally have little accelerator activity in the anesthetized rat even in the presence of enough atropine to block vagal reflexes.

The simplest explanation of these results seems to be as follows: pacemaker activity in the rat is under the influence of constant sympathetic tone which is increased by some anesthetics and is much greater during exercise. The relatively high saturation of the pacemaker with catechol amines masks the effect of added amines. Sympathetic tone is greatly reduced or abolished by reserpine treatment which brings heart rate closer to the intrinsic atrial frequency.

If this interpretation is true then the sympathetic tone during thiamine deficiency must be greater in each of the three groups (resting, anesthetized by pentobarbital sodium and exercising) since they each show a greater slowing than the normal rats in response to reserpine. A possible explanation is that sympathetic activity is increased by reflex mechanisms which partly compensate for the thiamine deficiency bradycardia. There is some evidence for increased sympathetic activity in other organs during thiamine deficiency (Ransford, '48) and Raab and Supplee ('44) showed an increase in the level of catechol amines in rat atria during thiamine deficiency.

It may be argued that the observed effects of reserpine are not due to its catechol amine depleting properties but to a more direct effect such as that observed by Innes and Krayer ('58). Two arguments may be put forward against this possibility. First, the slowing does not occur when the reserpine is given but only several hours afterwards. Maximal slowing is reached after about 24 hours when the level of catechol amines should be depleted to a level of about 1 to 5% of normal (Muscholl, '59; Schwartz and Lee, '60), at this time most of the reserpine has disappeared from the organism (Himwich, '58). Secondly, the fall of heart rate is paralleled by an increase in the response of the heart to adrenaline or noradrenaline injected intravenously. This is not due to a sensitization of the heart to catechol amines by the reserpine since reserpine itself in the isolated, depleted heart-lung preparation antagonizes the effects of catechol amines (Innes and Krayer, '58). This anomaly is explained if the pacemaker is nearly saturated with cardiac accelerator before reserpine treatments, thus obscuring the effects of added catechol amines.

The alternative possibility that reserpine acts by increasing vagal activity is ruled out by the absence of any acceleration after atropine or vagotomy in animals treated with reserpine.

The initial accelerating effect of reserpine on resting thiamine-deficient animals remains unexplained. It resembles the response seen by Krayer and Fuertes ('58) in the dog heart-lung preparation which they attributed to the release of catechol amines. An initial pressor response in intact animals has been observed in some species (Domino and Rech, '57). This response is inhibited by anesthesia.

Horita ('57) showed that the initial pressor response to reserpine in the dog is enhanced by cocaine and reduced by adrenalectomy so that it is probably associated with the release of catechol amines into the circulation. If this is so, it may mean that catechol amine stores are somewhat more labile during thiamine deficiency and thus are released more rapidly.

The evidence that the autonomic system opposes rather than causes the bradycardia of thiamine deficiency leads us back to the possibility that some accumulated metabolite is responsible. The increased concentration of metabolites usually associated with thiamine deficiency is insufficient to cause the bradycardia but Liang ('60) has recently shown that glyoxilic acid, a product of protein breakdown, accumulates during the deficiency. He believes that this may be responsible for many of the unexplained effects in-

cluding bradycardia[4] though this has not been tested quantitatively.

SUMMARY

The effects of ether, handling or spontaneous activity in rats all tend to obscure thiamine deficiency bradycardia by increasing the variability of heart rate. Conditions were described for the measurement of "resting" heart rate which has a low variability and may be used to detect thiamine deficiency at a relatively early stage. The extent to which the heart may accelerate above the resting level remained unimpaired in the early stages of thiamine deficiency bradycardia. At the same time reserpine had a relatively greater heart slowing effect on the thiamine-deficient animal. It was argued that the autonomic regulating mechanisms, far from being the cause of the bradycardia, tended to compensate for it. Unexpectedly, reserpine caused an initial tachycardia in thiamine-deficient unanesthetized animals before the slowing phase. This was not noted in normal animals and was largely obscured by anesthesia.

ACKNOWLEDGMENTS

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[4] Personal communication, Dr. C. C. Liang.

Effects of Potassium and Lysine Supplementation of Wheat Farina in Rat Diets[1]

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Potassium is the principal cation within tissue cells. It functions with bicarbonate, chloride and phosphate, and is intimately associated with the metabolism of glycogen and protein. Evidence has been presented to show: that potassium ions affect the permeability of cell membranes and the protein level of blood plasma (Conway and Hingerty, '48); that lysine plays a role in potassium deficiency in muscle (Eckel et al., '54; Iacobellis et al., '56); and that lysine and potassium play parallel substituting roles during the depletion of body proteins (Eliel et al., '52; Frost and Sandy, '53). Low levels of blood potassium have been observed in children suffering from kwashiorkor (Metcoff et al., '57; Senecal, '58). Potassium has been recommended by Sénécal ('58) and by Behar et al. ('58) in the treatment of kwashiorkor, mainly on the basis of results with high-protein diets fed to malnourished children.

Several investigators have observed an increased need for potassium by rapidly growing animals (Gillis, '48; Rome et al., '49; Heinicke et al., '56). Whitney and Bennett[3] reported that the effect of ACTH in lowering plasma nitrogen values was partially inhibited by increasing the potassium intake. The importance of higher potassium levels was also observed when high energy diets were fed to chickens (Leach et al., '59).

These observations suggest that a low level of potassium may have an important adverse effect in diets, and especially lysine-deficient diets. The level of potassium intake may be critical also when the rate of formation of body protein is increased due either to growth, or following protein depletion, or even in response to increased ingestion. Gershoff et al. ('59) studied the effects of different levels of potassium in the diet of weanling rats when varying amounts of lysine were added to supplement the cereal protein. He confirmed the positive effect of lysine on body weight gain, but failed to demonstrate a similar effect of potassium. Neither the body weight gains nor the concentrations of potassium, nitrogen and lysine in the muscles of animals receiving the lysine-deficient diets were affected by additions of potassium to the diet.

Barness et al. ('61) have recently reported that the supplementation of wheat farina with lysine, or with lysine and potassium, improved the nitrogen balance of malnourished children. They concluded that wheat protein supplemented with potassium and lysine is an adequate source of protein for growing infants, being close to milk protein in value for infants.

In a previous study in this laboratory (Harris and Burress, '59) wheat proteins, with and without lysine supplementation, were fed in the diets of weanling rats at 8 and 15% levels. These diets contained a salt mixture (Hegsted, '41) which furnished liberal amounts of all essential minerals, including potassium. The study reported here was designed to test the effects of different dietary levels of lysine

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[3] Whitney, J. E., and L. L. Bennett 1950 Inhibition of catabolic effect of ACTH on nitrogen metabolism by a high potassium diet. Federation Proc., 9: 134 (abstract).

and potassium, on the growth rate and body composition of weanling rats.

EXPERIMENTAL PROCEDURE

Four diets containing 8% of protein (wheat farina) were used in this research (table 1). Control diet B had the same composition as the control diet used previously by us (Harris and Burress, '59), except that glycine hydrochloride rather than sucrose was added to replace the lysine in the test diets to make them isonitrogenous. Diets C and D contained 0.31% of lysine hydrochloride, the amount found necessary by Harris and Burress ('59) to overcome the lysine deficiency of flour protein, and 4% of salt mixture (Hegsted, '41) which contributed 0.41% of potassium to the diet. Diets B and D contained 4% of salt mixture (Hegsted) but with the potassium salts replaced by the similar sodium salts. The sodium content of the four salts was equalized by the addition of sodium chloride. Thus, the protein of diets A and B was deficient in lysine (0.17%), whereas that of diets C and D was not (0.42%); and diets A and C were low in potassium content (0.075%), whereas diets B and D were not (0.485%).

A group of 66 male weanling rats (Charles River strain) were placed in individual wire-mesh cages with raised bottoms, and offered a standard stock diet for two days. They were then divided into 4 groups of 15 rats each with equal distribution according to weight and litter, and assigned to diets A to D. The diets and distilled water were offered ad libitum. The remaining 6 rats were sacrificed by decapitation and their carcasses were analyzed; they served as zero-day controls.

The body weight of each rat was recorded twice during the first week, then weekly until the end of the 4-week experiment. The amount of diet eaten was estimated twice weekly from records of food disappearance.

At the end of the experiment all rats were sacrificed, the livers from each group were pooled, the entrail-free carcasses were weighed, and all samples were placed at −40°C until analyzed. The livers were analyzed for moisture and lipid (ethyl ether extract) content. The carcasses were analyzed for moisture, protein (N × 6.25) and ash content according to AOAC ('60) procedures, and for potassium content according to the platinochloride precipitation method of Winton and Winton ('45). Because some of the rats in group A showed extensive alopecia, their carcasses were analyzed separately from those rats in group A which did not show this condition.

RESULTS

Body weight gains. During the 28-day period, the body weight gains (table 2) of groups C and D were significantly greater than the gains of groups A and B.[4] This confirms the results of many investigators who have observed that fortification of

[4] When considering as 2 × 2 factorial experiment, in this instance F = 76.5 with critical region 7.1 for 1 and 56 d.f. and 1% confidence interval (Li, '57).

TABLE 1

Composition of experimental diets

Constituent	Diet A	Diet B	Diet C	Diet D
Lysine·HCl	—	—	0.31	0.31
Glycine·HCl	0.31	0.31	—	—
Sucrose	27.69	27.69	27.69	27.69
Wheat farina	62.5	62.5	62.5	62.5
Corn oil	5.0	5.0	5.0	5.0
Salt mixture[1]	—	4.0	—	4.0
Salt mixture (potassium-free)	4.0	—	4.0	—
Vitamin mixture[2]	0.5	0.5	0.5	0.5
Totals	100.0	100.0	100.0	100.0
Total lysine content	0.17	0.17	0.42	0.42
Total potassium content	0.075	0.485	0.075	0.485

[1] Hegsted ('41).
[2] For composition of vitamin mixture see Harris and Burress ('59).

wheat protein with lysine has a significant effect on the growth of immature rats.

The differences in the body weight gains of group A versus B, and C versus D were not significant ($F = 0.194$), indicating that the potassium supplement *per se* had no effect on the growth of rats. This confirms a previous observation on rats by Gershoff ('59) who used diets containing 15% rather than 8% of protein, but differs from results obtained by Barness et al. ('61) on children who noted that the nitrogen balance was improved by the addition of potassium to farina diets.

Protein utilization. It is evident from table 2 that the utilization of the dietary protein was improved by lysine supplementation but not by potassium supplementation. Lysine, but not potassium, improved the protein efficiency ratio (gram of body protein gain per gram of diet protein eaten) of the wheat farina.

Liver composition. The livers of the rats fed the lysine-supplemented diets were heavier and contained much less lipid than the livers of the rats fed unsupplemented wheat farina (table 3). This is additional evidence that diets containing lysine-deficient protein will cause fatty livers (Harper, '58; Harris and Burress, '59) which are preventable by lysine supplementation. A mild lipotropic effect resulted from the addition of potassium to the lysine-deficient diet, but not to the lysine-sufficient diet.

Carcass composition. The percentage of moisture and nitrogen in the carcasses from the 4 groups of rats (see table 4) were not significantly different. The ash content was lower in those 6 rats fed diet A (low potassium, low lysine) that showed extensive alopecia during the experiment, than in the 9 rats of this group that did not lose hair. The low potassium content of the carcasses of these rats indicates that the alopecia may have been related to a disturbance in potassium metabolism. The carcass-potassium levels were significantly (> 30%) lower in the groups (A, C) fed the low potassium diet than in the groups (B, D) supplied with liberal amounts of dietary potassium. Possibly the deficiency of lysine in the diet of group A acted as a stress factor in potassium metabolism, for the carcasses of these rats contained somewhat less potassium than

TABLE 2

Diet consumption, body weight and body protein gains during 4 weeks (av./rat/28 days)

	Diet A (no addition)	Diet B (added potassium)	Diet C (added lysine)	Diet D (added potassium and lysine)
Body weight gain, gm	9.4	8.4	21.7	24.0
Body protein gain, gm	0.6	0.8	3.1	2.6
Diet eaten, gm	152.0	158.7	171.4	169.5
Diet protein eaten, gm	12.2	11.1	13.7	13.6
$\frac{\text{Body protein gain}}{\text{Diet protein eaten}} \times 100$	4.7	6.9	22.4	19.2
Potassium eaten				
as farina	0.11	0.10	0.13	0.13
as salt mixture	—	5.55	—	6.78
total, gm	0.11	5.65	0.13	6.91

TABLE 3

Composition of livers (average per rat)

	Diet A (no additions)	Diet B (added potassium)	Diet C (added lysine)	Diet D (added potassium and lysine)
Total weight, gm	2.7	2.6	4.2	4.2
Solids, %	29.5	31.2	27.2	27.2
Lipids, wet basis, %	7.08	8.77	3.94	4.00
Lipids, dry basis, %	24.0	28.1	14.5	14.7

TABLE 4

Composition of carcasses (average/rat)

	0-Day control rats	Diet A	Diet A group divided		Diet B	Diet C	Diet D
			With alopecia	Without alopecia			
Initial body weight, gm	—	43.4	—	—	43.2	43.3	43.1
Final body weight, gm	—	52.8	—	—	51.7	65.1	67.1
Final weight entrail-free carcasses, gm	—	43.4	43.4	43.4	43.1	57.4	56.4
Total solids, %	33.4	34.7	35.6	34.3	35.5	34.4	34.6
N × 6.25, wet basis, %	18.5	16.3	15.8	16.5	16.9	17.0	15.8
N × 6.25, dry basis, %	55.3	46.8	44.3	48.1	47.5	49.5	45.5
Ash, dry basis, %	—	11.9	10.2	12.7	12.6	11.7	11.8
K, in ash, %	—	10.3	8.5	11.1	12.2	10.3	12.4
K, wet basis, %	—	0.39	0.37	0.41	0.55	0.41	0.51
K, dry basis, %	—	1.12	1.08	1.14	1.54	1.20	1.46
Total K/rat carcass, gm	—	0.17	0.13	0.20	0.24	0.18	0.27

group C. The levels of potassium in rat carcasses, as observed in this study (table 4), are in agreement with those observed by Muntwyler et al., ('58). Holliday and Segar ('57) observed a similar (15 to 18%) decrease of potassium in the muscle tissues of rats fed potassium-deficient diet for 28 days. Eckel et al. ('54) observed an effect of lysine in potassium-deficient animals equivalent to some 10 mEq of cation per liter of intracellular water, and they suggested that lysine initially replaces the potassium lost from cells prior to significant gain in intracellular sodium. When the sodium intake was not restricted, as in our experiment, a decrease in tissue potassium content was accompanied by a high exchange rate of Na for K, and the effect of lysine could not longer be observed.

SUMMARY AND CONCLUSIONS

Four diets containing wheat farina as the sole source of protein (8%) were supplemented with zero and 0.31% of L-lysine hydrochloride and/or with salt mixtures containing zero and 0.41% of potassium, and fed ad libitum to 4 groups of weanling rats. The animals were decapitated at the end of 28 days; their livers were analyzed for moisture and lipid content, and their carcasses were analyzed for moisture, ash and potassium content.

The amount of carcass protein synthesized per gram of protein eaten was significantly improved by the addition of lysine to the diet, but not by the addition of potassium.

"Fatty livers" were observed in the rats fed the lysine-deficient diets, but not in the rats receiving the lysine-sufficient diets.

The groups fed diets deficient in lysine, in potassium and in both lysine and potassium contained 10, 35 and 40% less potassium in their carcasses, respectively, than the group fed the control diet adequate in both these nutrients.

The synthesis of carcass protein by weanling rats was not significantly affected by the potassium content of the farina diet containing 8% protein.

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Effect of Vitamin D on the Utilization of Beryllium, Magnesium, Calcium, Strontium and Barium in the Chick[1]

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The function of vitamin D in the animal body is still not wholly understood. That it plays an important role in the metabolism of calcium, however, has been known for many years and much of the recent work in this field has been aimed at clarifying this relationship. It is now generally agreed that vitamin D exerts one of its principal effects on calcium metabolism by promoting the transfer of dietary calcium across the intestinal wall into the bloodstream.

Few attempts have been made to study systematically the effect of vitamin D on other cations. There is evidence to suggest, however, that vitamin D may possibly play a much more important role in the overall mineral economy of the animal than has hitherto been suspected. For example, in balance studies in rats Meintzer and Steenbock ('55) observed that rations low in vitamin D slightly depressed magnesium absorption. In an exhaustive study of lead metabolism, Sobel and his associates (Sobel et al., '40; Sobel and Burger, '55) observed in feeding trials with rats that vitamin D increased markedly the levels of lead in both blood and bone. The relation of vitamin D and strontium metabolism has also been investigated by a number of workers and there is considerable evidence to indicate an effect of vitamin D on uptake of dietary strontium (Greenberg, '45; Patrick and Bacon, '57; Mraz and Bacon, '60).

The following experiments were undertaken with a view to examining further the effect of vitamin D on the utilization of a number of different cations. The present communication describes results of experiments with chicks using beryllium, magnesium, strontium, calcium and barium.

MATERIALS AND METHODS

Preparation and dosing of chicks. The chicks used in these experiments were New Hampshire/Rhode Island Red cross (experiments 129 to 133, 133A) and White Rock (experiments 138, 140). The day-old chicks received a low-calcium modification (Migicovsky and Emslie, '47) of the AOAC ('40) rachitogenic diet until body stores of vitamin D were depleted and signs of rickets were observed, usually at three to 5 weeks of age. In some early experiments the presence and severity of rickets were assessed by x-ray. One half of the birds were dosed orally with a colloidal suspension of vitamin D_3 in water at the rate of 2000 I.U. per bird. The dosed birds were also fed a diet supplemented with vitamin D_3 at the rate of 1000 I.U. per 100 gm of feed. These groups were then further subdivided prior to isotope dosing into smaller treatment groups containing 10 to 20 birds. Forty-eight hours later, after an overnight fast, all birds were given an oral, or subcutaneous double tracer dose of Ca^{45} with Be^{7}, Sr^{90}, or Ba^{131}. γ-Emitting isotopes of the latter elements were chosen to facilitate their determination in the presence of Ca^{45}. With Mg^{28}, which is both a β- and γ-emitter, the tracers were not dosed simultaneously

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but were given to two separate lots of birds chosen randomly from within each vitamin D treatment group. The radiation dose varied from two to 4 μc depending on the specific activity of the sample, care being taken to see that the combined cation dose did not exceed 2.0 mg per chick. The birds were killed with chloroform anaesthesia 24 hours after the isotope dose and one tibia was removed for assay. Bones containing Sr and Ca or Ba and Ca were ashed prior to assay, those containing Be or Mg (due to the paucity of data on the stability of Be- and Mg-phosphates) were dissolved directly in concentrated hydrochloric acid.

Measurement of Be^7, Mg^{28}, Sr^{90} and Ba^{131}. γ-Emission of dosing solutions and solutions of bone or bone-ash were measured in a Nuclear Chicago model DS5-5 scintillation well counter (sodium iodide thallium-activated crystal) connected through a model 1810 γ-ray spectrometer. Appropriate corrections were made for background radiation.

Measurement of Ca^{45}. β-Emission of calcium oxalate pads, prepared from dosing solutions and solutions of bone or bone-ash, were measured in a Nuclear Chicago model 192A gas flow counter, appropriate corrections being made for background radiation and loss due to self absorption.

Presentation. In the present experiments we were interested in investigating two separate but related aspects of vitamin D action: (1) the effect of vitamin D status on uptake from the dose and deposition in bone of the individual elements under study; and (2) the effect of vitamin D status on the uptake and deposition of each element *relative* to calcium. In the first case deposition was expressed in terms of absolute counts per minute per bone and differences between groups have been treated by accepted statistical procedures (Snedecor, '56). In the second case bone deposition of individual elements, relative to that of calcium, was calculated using the expression (Comar et al., '56).

$$\text{O.R.}_{(\text{bone-dose})} = \frac{\dfrac{\text{counts per minute per bone X}}{\text{counts per minute per bone Ca}}}{\dfrac{\text{counts per minute per dose X}}{\text{counts per minute per dose Ca}}}$$

where X = individual element under study; O.R. = observed ratio, a measure of the discrimination against X, relative to calcium, in movement from dose to bone.

RESULTS

The influence of vitamin D on the levels of the various cations appearing in bone after oral administration is shown in table 1. The results indicate in every case that supplementing with vitamin D 48 hours before dosing with isotope caused a significant increase in the levels of radioactivity present.

In table 2 observed ratios based on the same data from which table 1 was derived are presented. Relative to calcium, there were considerable differences in the facility with which the individual cations entered bone. Barium was metabolized as well as calcium, but beryllium was poorly metabolized. Strontium was somewhat less well metabolized than barium, whereas magnesium was intermediate between strontium and beryllium. Observed ratios in both vitamin D-supplemented and non-supplemented groups dosed with barium and with strontium were similar. Supplementing with vitamin D, however, significantly reduced the ratios in groups dosed with magnesium and beryllium.

Dosing with vitamin D did not increase the levels of radioactivity in bone after subcutaneous administration of isotope (table 3). On the contrary, the bones from groups supplemented with vitamin D appeared to be regularly lower in counts than those from non-supplemented groups, although only in the case of magnesium was this observed to be a statistically significant trend.

The ratios presented in table 4 are based on the same data from which table 3 was calculated. These results suggest that although barium and strontium moved from blood to bone at a rate similar to that of calcium there was considerable discrimination against both beryllium and magnesium. In no instance did vitamin D status appear to exert any significant influence on the magnitude of the observed ratio (O.R.) in chicks dosed with isotope subcutaneously.

DISCUSSION

The effect of vitamin D on calcium metabolism has been subject to exhaus-

TABLE 1

Effect of vitamin D on levels of radioactivity in chick tibia following administration of isotopes orally

Exp. no.	Element	Dose × 10^3	Vitamin D treatment		Significance of difference P
			Non-supplemented	Supplemented	
		cpm	*cpm/tibia*	*cpm/tibia*	
129	Sr^{85}	106	810 ± 90[1]	3630 ± 310[1]	< 0.01
	Ca^{45}	2100	20390 ± 770	74550 ± 5280	< 0.01
129A	Sr^{85}	500	3680 ± 230	18000 ± 1170	< 0.01
	Ca^{45}	2100	20370 ± 1790	85300 ± 6250	< 0.01
129B	Sr^{85}	2050	16440 ± 1850	42500 ± 2620	< 0.01
	Ca^{45}	1900	19240 ± 1800	46880 ± 2520	< 0.01
	Ba^{131}	770	7610 ± 2620	25560 ± 2250	< 0.01
	Ca^{45}	1900	18250 ± 1660	52750 ± 2200	< 0.01
132	Be^{7}	1200	1590 ± 192	2265 ± 178	< 0.05
	Ca^{45}	2000	21090 ± 4190	46910 ± 2860	< 0.01
	Sr^{85}	2850	18460 ± 820	29770 ± 1640	< 0.01
	Ca^{45}	4200	32840 ± 2270	51100 ± 3370	< 0.01
133	Be^{7}	1100	1250 ± 170	1780 ± 108	< 0.05
	Ca^{45}	4000	40000 ± 3260	108000 ± 1660	< 0.01
	Ba^{131}	1450	14480 ± 1840	35920 ± 1920	< 0.01
	Ca^{45}	4000	43690 ± 4170	111540 ± 3370	< 0.01
138	Mg^{28}	40	550 ± 25	820 ± 70	< 0.01
140	Mg^{28}	48	420 ± 20	620 ± 65	< 0.05
	Ca^{45}	1200	35750 ± 3370	80250 ± 3130	< 0.01

[1] Mean ± standard error of mean.

TABLE 2

Values of observed ratios calculated from original data from table 1

Exp. no.	Elements	Observed ratio[1]		Significance of difference P
		Non-supplemented	Supplemented	
129	Sr/Ca	0.72 ± 0.03	0.79 ± 0.06	NS[2]
129A	Sr/Ca	0.74 ± 0.03	0.82 ± 0.04	NS
129B	Sr/Ca	0.67 ± 0.04	0.70 ± 0.04	NS
	Ba/Ca	0.92 ± 0.07	0.98 ± 0.09	NS
132	Be/Ca	0.13 ± 0.02	0.06 ± 0.01	< 0.05
	Sr/Ca	0.81 ± 0.04	0.81 ± 0.04	NS
133	Be/Ca	0.11 ± 0.01	0.05 ± 0.01	< 0.01
	Ba/Ca	0.93 ± 0.05	1.01 ± 0.03	NS
140	Mg/Ca	0.29 ± 0.03	0.19 ± 0.01	< 0.05

[1] Mean ± standard error of mean.
[2] Indicates not significant.

tive investigation and there is a large body of experimental evidence to show that vitamin D plays an important role in the absorption of this element (Keane et al., '56). A similar influence is exerted on strontium uptake (Mraz and Bacon, '60) and at least one report suggests that magnesium likewise is affected (Meintzer and Steenbock, '55). The work reported in this communication was carried out in the chick using an experimental design which has been in routine use in this laboratory for many years in studies on calcium metabolism. The experiments undertaken were all of a short-term nature and no

TABLE 3

Effect of vitamin D on levels of radioactivity appearing in chick tibia following administration of isotopes subcutaneously

Exp. no.	Element	Dose $\times 10^3$	Vitamin D treatment		Significance of difference P
			Non-supplemented	Supplemented	
		cpm	*cpm/tibia*	*cpm/tibia*	
133A	Be^7	1100	14800 ± 1720[1]	13980 ± 1890[1]	NS[2]
	Sr^{85}	185	6990 ± 880	6490 ± 910	NS
	Ba^{131}	300	11780 ± 1030	10990 ± 1480	NS
	Ca^{45}	580	22200 ± 1940	20080 ± 1160	NS
140	Mg^{28}	24	607 ± 20	507 ± 25	< 0.01
	Ca^{45}	600	38100 ± 680	37100 ± 800	NS

[1] Mean ± standard error of mean.
[2] Indicates not significant.

TABLE 4

Values of observed ratios calculated from original data from table 3

Exp. no.	Elements	Observed ratio[1]		Significance of difference P
		Non-supplemented	Supplemented	
133A	Be/Ca	0.34 ± 0.02	0.36 ± 0.03	NS[2]
	Sr/Ca	0.99 ± 0.03	1.01 ± 0.03	NS
	Ba/Ca	0.97 ± 0.05	0.99 ± 0.04	NS
140	Mg/Ca	0.39 ± 0.03	0.34 ± 0.01	NS

[1] Mean ± standard error of mean.
[2] Indicates not significant.

attempt was made to study the possible long-term effects of the vitamin.

The results demonstrate that in vitamin D-supplemented chicks the levels in bone of all the cations studied were significantly increased following their administration by mouth. That this was due to an effect of vitamin D on the absorptive processes was strongly suggested by the fact that vitamin D caused no such increase in count following administration of isotopes by the subcutaneous route. This is in close conformity also with findings obtained previously by Migicovsky and Nielson ('51) and Migicovsky and Jamieson ('55) in studies on calcium absorption.

That vitamin D should have increased the uptake of every element suggests that it may possibly play a much more general role in mineral metabolism than has formerly been suspected. Such a possibility clearly raises questions as to the extent of this effect and the practical importance of the role played by vitamin D in the overall mineral economy of the organism.

From a consideration of the values of the observed ratios found after administration of the isotopes subcutaneously, it is clear that the rate of movement of the various cations from blood to bone, relative to that of calcium, was not influenced by vitamin D treatment. Values of observed ratios obtained after administration of the isotopes orally, however, were significantly decreased by vitamin D treatment in the case of beryllium and magnesium, due presumably to the rate of movement of these cations across the gut wall being less markedly influenced than calcium by vitamin D supplementation.

The significance of the finding in the orally dosed groups that the observed ratios Be/Ca < Mg/Ca < Sr/Ca < Ba/Ca is not clear. It is suggested that these ratios may possibly indicate the relative degree of affinity of these cations for a specific surface during the absorptive process. This possibility is being further investigated.

SUMMARY

The influence of vitamin D on the utilization of Ca, Be, Mg, Sr and Ba after administration of isotopes of these elements orally and subcutaneously to rachitic chicks is reported.

The uptake of all of these elements from an oral dose, as measured by deposition in bone, was significantly increased by vitamin D. When the elements were dosed subcutaneously, however, the only effect produced by vitamin D was on magnesium, the level of which was decreased in bone.

When given orally there was considerable discrimination, relative to calcium, against beryllium and magnesium and also a smaller discrimination against strontium. Barium, however, appeared to behave like calcium. Vitamin D significantly increased discrimination against beryllium and magnesium but was without effect on strontium and barium.

When the elements were administered subcutaneously, there was also considerable discrimination against the movement of beryllium and magnesium into bone but not against strontium and barium. Vitamin D caused no change in this pattern.

The possibility is suggested that vitamin D may play a considerably broader role in mineral economy than has hitherto been recognized.

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Vitamin E Saturation Test in Coccidiosis-Infected Rabbits[1,2]

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Earlier studies (Diehl, '60) have shown that young rabbits infected with *Eimeria stiedae* had significantly lower tissue vitamin E levels than did noninfected controls. The question arose as to the mechanisms by which coccidiosis infection might lead to low vitamin E reserves. One obvious possibility appeared to be malabsorption of the dietary vitamin E due to biliary obstruction. Jaundice is known to occur in the advanced stage of hepatic coccidiosis (Smetana, '33) and obstruction of the bile duct has been shown to interfere with intestinal absorption of tocopherol in the rat (Klatskin and Tisdale, '57; Greaves and Schmidt, '37).

Tocopherol saturation tests, with both oral and parenteral administration of vitamin E preparations to infected and noninfected rabbits, were used in the present investigation to study this question.

METHODS

It has been pointed out earlier (Diehl, '60) that it is very difficult to obtain commercially rabbits that are reliably free of coccidiosis. A colony of White New Zealand rabbits was started in an isolation area from coccidiosis-free pregnant does.[3] The does received 0.04% of sodium sulfaquinoxaline in the drinking water for three-day periods alternating with two-day periods of plain water. The same regimen was used for the young rabbits born in this colony. All animals were kept in wire-bottom cages which were washed and sterilized frequently. Young rabbits were transferred to individual cages as soon as they were old enough to be weaned.

Four of these animals were used in the vitamin E-saturation experiment when they were 10 weeks old. Six other animals were infected, by stomach tube, with a suspension of approximately 50,000 sporulated *E. stiedae* oocysts when they were 7 weeks old. They received no sulfaquinoxaline thereafter and were used in the vitamin E-saturation experiment when they were 10 weeks old. The attendant of the coccidiosis-free colony had no direct contact with infected rabbits. All animals received commercial rabbit chow ad libitum, which contained 0.8 mg of α-tocopherol per 100 gm.

In the vitamin E saturation experiment 1 mg of α-tocopherol per 100 gm of body weight was administered at the same hour on 4 subsequent days. One half of the animals received the dose orally by pipette, as *d*-α-tocopheryl acetate in corn oil (solution containing 20 mg of α-tocopherol per ml). The other half of the animals received by intraperitoneal injection an aqueous solution of *d*-α-tocopheryl-polyethylene-glycol-1000 succinate[4] containing 10 mg of α-tocopherol per ml. Four milliliters of blood were collected by heart puncture from each animal one hour before the first tocopherol was given (day zero) and every 24 hours thereafter. Feces were collected daily and stored in closed containers under refrigeration until vitamin E analyses could be carried out (not longer than 5 days). The

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experiment was terminated 7 days after the first tocopherol administration. The animals were decapitated and livers removed for vitamin E analysis. Microscopic examination of the gall bladder contents showed merozoites and oocysts of *E. stiedae* only in the infected animals. Gross examination of the livers showed a moderate degree of lesions in infected rabbits. The effect of the daily bloodletting during a 7-day period on the hematology of the rabbit was observed in a preliminary experiment with one animal. The hemoglobin content dropped from 12.3 to 11.5 gm per 100 ml and the erythrocyte count from 6.15 to 4.0 million per µl. The serum vitamin E level remained substantially unchanged.

The 10 rabbits used in the saturation experiments were offspring of 4 different litters. They were distributed randomly between the two treatment groups in order to minimize the possible influence of genetic factors on the results of the experiments.

Total tocopherols of serum were determined by the method of Rindi ('58) and liver tocopherols by the method of Swick and Baumann ('52). Feces from each animal were pooled for days zero to 5 and for days 6 plus 7, freeze-dried, weighed and pulverized. Duplicate 5-gm samples were extracted in large centrifuge tubes with 25 ml of boiling ethanol for 10 minutes, centrifuged and washed twice with cold ethanol. The combined ethanol extracts were heated to boiling, 10 ml of a solution containing 2% potassium hydroxide and 0.5% *p*-acetylaminophenol in ethanol, and 5 ml of water were added. The mixture was boiled for 25 minutes. Thirty milliliters of water were added, the solution was saturated with sodium sulfate, and twice extracted with 20-ml portions of petroleum ether. The two petroleum ether fractions were combined and dried over sodium sulfate for two hours and brought up to a volume of 40 ml with petroleum ether. Ten-milliliter aliquots were evaporated *in vacuo*. The residue was dissolved in benzene. Subsequent determination of total tocopherols by the Emmerie-Engel reaction after purification on a Florex column was carried out as described for tissue tocopherol determinations (Swick and Baumann, '52).

RESULTS AND DISCUSSION

The response of serum tocopherol levels to the tocopherol administrations (fig. 1) confirms the earlier report (Diehl, '60) of low vitamin E reserves in the coccidiosis-infected rabbits. Statistical evaluation of the serum tocopherol data by the *t* test (Snedecor, '56) shows that the differences between the means of infected and noninfected groups seen on day zero are not significant. The differences observed after three tocopherol administrations are significant ($P < 0.05$). The fact that serum tocopherol levels of infected rabbits after parenteral administration are not substantially higher than after oral administration, speaks against a defect of intestinal absorption in coccidiosis infection. This finding is confirmed by the fecal recovery data. Considerable individual variation is shown in table 1, but certainly it can be said that tocopherol excretion after oral administration is not higher in infected animals than in controls. Fecal tocopherol excretion was actually lower in the infected animals, both after oral and after intraperitoneal administration of the vitamin. This may be considered as an additional indication of low tocopherol reserves in the infected animals. Liver tocopherol was determined in 4 of the 6 infected animals and in the 4 controls, and was found to be lower in the infected groups (table 2).

In view of reports that some of the reducing material measured by the Emmerie-Engel reaction in liver extracts is not tocopherol (Bieri et al., '60; Crider et al., '60), it might be argued that the differences in Emmerie-Engel positive material observed in infected and noninfected animals are perhaps not due to differences in tocopherol levels. The earlier report (Diehl, '60), however, has shown that no Emmerie-Engel positive material was found in livers of some severely infected animals. We have repeatedly confirmed this observation since then. Also, the fact that in the present study infected animals responded to repeated doses of authentic tocopherol with a relatively small increase in Emmerie-Engel reactive material in the blood, can

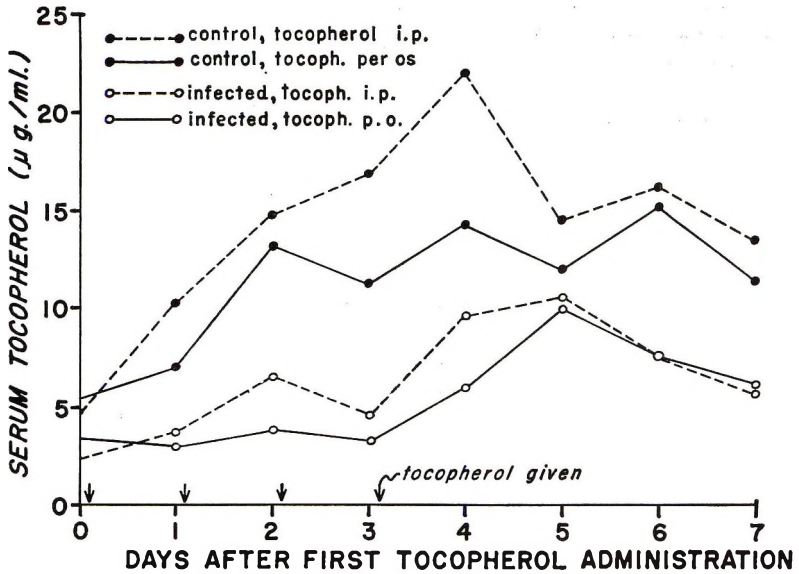

Fig. 1 Serum tocopherol levels of infected and control rabbits during saturation test. Each experimental point represents the mean for groups of three infected or two control rabbits.

TABLE 1

Total tocopherol excretion in feces during saturation test

Condition of rabbits and type of tocopherol administration	Tocopherol recovery, % of intake[1]			
	Days 1–5		Days 6–7	
		Av.		*Av.*
Noninfected rabbits, control				
Intraperitoneal injection	14.8; 28.2	21.5	22.8; 42.8	32.8
Oral dose	27.4; 46.6	37.0	34.6; 60.6	47.6
Infected rabbits				
Intraperitoneal injection	10.2; 2.8; 3.5	5.5	13.9; 3.2; 3.8	7.0
Oral dose	16.3; 10.6; 5.4	10.4	17.1; 11.8; 7.2	12.0

[1] Intake is the sum of oral or parenteral tocopherol plus tocopherol consumed with the diet. The amount of tocopherol consumed with the diet by each rabbit in one day, as estimated by weighing food cups, was less than 2% of the daily amount administered intraperitoneally or orally.

TABLE 2

Tocopherol content of rabbit livers at termination of the saturation test

Condition of rabbits and type of tocopherol administration	Tocopherol/gm liver		Tocopherol in whole liver/100 gm body weight	
	μg	*Av., μg*	*μg*	*Av., μg*
Noninfected rabbits, control				
Intraperitoneal injection	15.4; 19.2	17.3	50.7; 64.5	57.7
Oral dose	10.1; 14.0	12.3	33.7; 54.3	44.0
Infected rabbits				
Intraperitoneal injection	12.0; 6.2	9.1	55.6; 17.8	36.7
Oral dose	10.6; 5.1	7.8	41.5; 14.9	28.2

be taken as an indication that tocopherol levels are low in the infected animals.

Two possible mechanisms for the effect of coccidiosis infection on vitamin E reserves remain. (A) The disease impairs the ability of the liver to take up and/or store vitamin E. Since the liver is an important storehouse for the vitamin [Mason, ('42) has shown that changes in dietary intake are most directly reflected in liver vitamin E levels], any reduction in the capacity of the liver to handle vitamin E may well be reflected in lower tocopherol levels in other tissues. In a tocopherol saturation test much of the administered tocopherol would be used to replete tissue tocopherol levels to normal, resulting in low fecal tocopherol recovery and in a flat serum tocopherol curve. It may be recalled that Moore ('57) attributed the very low vitamin A content of cirrhotic livers to the replacement of many of the liver cells by fibrous tissue, which has little affinity for vitamin A. A similar situation may exist for vitamin E in coccidiosis-infected livers.

(B) Hepatic coccidiosis leads to increased utilization or destruction of vitamin E, either through a direct effect of the parasitic organism, or indirectly, through an effect of the injured host tissue. In both cases low tissue and serum tocopherol levels and low fecal recovery in a saturation test would be expected. Direct effects or other parasites on the vitamin reserves of the host have been described, namely, *Diphyllobothrium latum*, a cestode, has been shown to cause a macrocytic anemia in the human host by competing for his vitamin B_{12} (Von Bonsdorff, '56). Inhibition of organisms which inactivate or render vitamins unavailable to the host has been suggested as one explanation for the nutritional effect of antibiotics. Stokstad ('56) reviewed a number of papers which reported increased vitamin A levels in serum and liver of animals treated with antibiotics. Nason ('58) has made the suggestion that certain quinoline derivatives which act as antimalarial compounds may be antagonists of vitamin E and that they may be exerting their antimalarial action by occupying the tocopherol sites in the parasitic organism. A requirement of micro-organisms for vitamin E, however, has not been established and so far only some chlorophyll - containing micro - organisms have been shown to contain α-tocopherol (Green et al., '59).

SUMMARY

1. Rabbits infected with *Eimeria stiedae* and noninfected rabbits received repeated oral or parenteral administrations of vitamin E. Serum, feces and liver were analyzed for vitamin E.

2. The earlier observation that hepatic coccidiosis leads to low vitamin E reserves of the host animal has been confirmed.

3. The results exclude intestinal malabsorption of the vitamin as the cause for the low tissue vitamin E levels in the infected rabbits.

4. Other possible mechanisms for the effect of coccidiosis on the vitamin E reserves of the host are discussed.

ACKNOWLEDGMENT

The authors wish to acknowledge the valuable assistance of Mrs. T. E. Ashcraft and Charles Weir.

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Influence of Dietary Protein Level on Serum Protein Components and Cholesterol in the Growing Chick

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The influence of dietary protein on serum proteins has been demonstrated in the rat and dog (Allison, '55; Whipple, '48). The changes observed in total serum protein in the rat have been shown to result primarily from an alteration of the albumin level with little or no change in the globulin fractions; these variations, thereby, resulting in a marked alteration of the albumin to globulin ratio (A/G).

The influence of diet on serum protein components of the chick has received little attention. In a recent study (Leveille et al., '60), it was shown that dietary fat and cholesterol exert little influence on the serum proteins of the chick; the dietary protein level, on the other hand, markedly affected the level of total serum proteins and albumin.

The influence of dietary protein on serum cholesterol in the chick has received considerable attention. It has been demonstrated that the serum cholesterol level can be decreased by increasing dietary protein (Leveille and Fisher, '58; Leveille et al., '60; Johnson et al., '58; Nishida et al., '58; Kokatnur et al., '58; Nikilla and Ollila, '57).

The present study was designed to further study the influence of dietary protein level on serum protein components and serum cholesterol in the growing chick, as well as to obtain data on the changes which might result in plasma volume as a consequence of feeding various protein levels.

EXPERIMENTAL

Day-old male Hy-line Leghorn chicks were fed for one week with a practical starting ration, at which time they were divided into 4 groups of 14 chicks each. The chicks were divided in such a manner that the average initial weight (weight at 7 days of age) of each group was identical, 59 gm. The percentage composition of the basal diet was as follows: sesame oil meal (46.8% protein), 21.35; corn oil, 5.00; salt mixture, 4.00;[1] vitamin mixture, 2.20;[2] L-lysine·HCl (99%), 0.90; starch, 23.04; and sucrose to 100. The various protein levels (10, 15, 20 and 25% of the diet) were obtained by the addition of sesame oil meal to the diet at the expense of sucrose.

The chicks were fed the experimental diets for three weeks in electrically heated cages with raised wire floors. Feed and water were supplied ad libitum. The birds were weighed and food consumption determined weekly. At the end of the experimental period, 10 chicks randomly selected from each group were bled by cardiac puncture. The blood was allowed to clot and the serum was decanted after centrifugation. The 4 remaining chicks from each treatment were used for plasma volume determinations.

Total serum protein was determined by the biuret method of Gornall et al. ('49) and serum cholesterol by a modification of the method of Zlatkis et al. ('53) using the stable iron reagent of Rosenthal et al. ('57). Paper electrophoresis was carried out with a Beckman-Spinco Durrum-type cell employing a barbiturate buffer (pH 8.6; ionic strength 0.075). Twenty lambda of serum were applied to each strip and the separation was carried out at a temperature of 20°C for 20 to 24 hours; after separation the strips were dried and stained with

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[1] Supplies per kilogram of diet: (in grams) $CaCO_3$, 3.00; $Ca_3(PO_4)_2$, 18.00; K_2HPO_4, 9.00; $MgSO_4$, 1.25; ferrous gluconate, 2.24; NaCl, 8.80; and (in milligrams) $ZnSO_4 \cdot 7H_2O$, 60; KI, 40; $CuSO_4$, 20; H_3BO_3, 9; $CoSO_4 \cdot 7H_2O$, 1.00; $MnSO_4 \cdot H_2O$, 650.

[2] Vitamin Diet Fortification mixture purchased from Nutritional Biochemicals Corporation, Cleveland.

methanolic bromophenol blue. The relative amount of protein in the various fractions on the stained strips was determined by use of an integrating photometric scanner (Analytrol). Absolute concentrations of the various fractions were estimated by relation to the total serum protein levels.

Plasma volume was determined by the Evans blue dye dilution technique as described by Medway and Kare ('59).

RESULTS

The influence on body weight of the protein levels used is shown in table 1 and graphically in figure 1. A linear response was obtained in body weight for the three lowest levels of protein fed (10, 15 and 20%). The observed data showed an excellent fit to the least squares line drawn through these points. There was no detectable difference in body weight between the animals fed the 20 and 25% levels of protein; therefore, the protein requirement for maximum growth under these conditions can be taken as the point where the least squares line, calculated for the body weight at the three lower protein levels fed, intersects a horizontal line drawn at the mean body weights of the groups fed the 20 and 25% protein levels. This requirement is 20.5% of protein (fig. 1).

Serum protein was related to dietary protein at all 4 levels fed (fig. 2a). Serum cholesterol levels were inversely related to the dietary protein levels, (fig. 2b). Although no growth response was noted between the 20 and 25% protein levels, there was a difference in serum protein and cholesterol concentrations.

The results of the electrophoretic analyses and plasma volume data are presented in table 1, along with final body weight and serum protein data. There were no differences between the various treatment groups with respect to the alpha or beta + gamma protein fractions or in the plasma volume when expressed as a percentage of body weight. The increases in total serum protein observed at each increasing level of dietary protein can be accounted for by increases in the albumin fraction. As a consequence of the higher albumin levels and relatively stable globulin levels, observed, the albumin to globulin ratio increased as dietary protein was increased (table 1).

TABLE 1

Influence of dietary protein on serum protein components, body weight, plasma volume and serum cholesterol of the growing chick

Dietary protein level	4-Week body weight	Total serum protein	Albumin	Globulin		A/G[1]	Plasma volume	Serum cholesterol
				Alpha	Beta + gamma			
%	*gm*	*gm/100 ml*	*gm/100 ml*	*gm/100 ml*			*% of body weight*	*gm/100 ml*
10	156 ± 33[2]	2.33 ± 0.32	1.22 ± 0.24	0.52 ± 0.11	0.55 ± 0.22	1.14	—	297 ± 27
15	221 ± 40	2.60 ± 0.49	1.50 ± 0.38	0.50 ± 0.08	0.61 ± 0.21	1.35	7.2	244 ± 33
20	316 ± 27	2.80 ± 0.11	1.78 ± 0.10	0.46 ± 0.05	0.56 ± 0.05	1.74	6.8	216 ± 20
25	323 ± 21	3.06 ± 0.21	1.97 ± 0.19	0.50 ± 0.09	0.59 ± 0.06	1.81	7.1	185 ± 19

[1] Albumin to globulin ratio.

[2] Mean ± standard deviation.

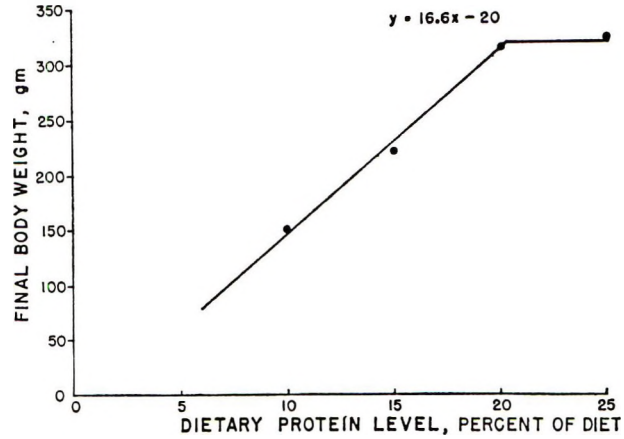

Fig. 1 The influence of dietary protein level on body weight of the growing chick.

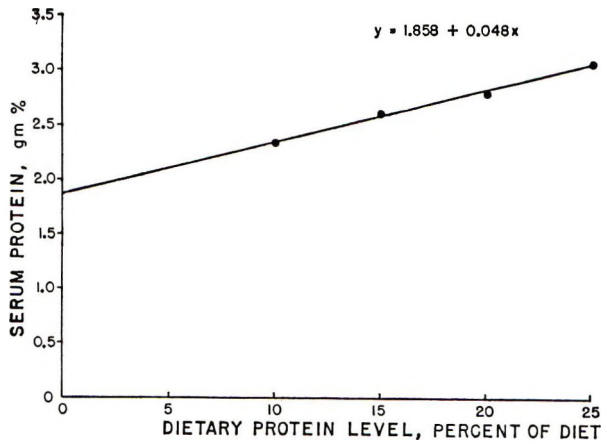

Fig. 2a The influence of dietary protein level on total serum proteins.

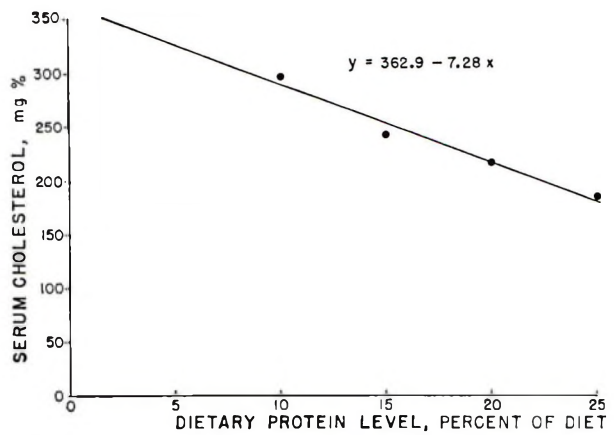

Fig. 2b The influence of dietary protein on total serum cholesterol in the growing chick.

DISCUSSION

The data presented in this report demonstrate that the serum protein concentration is influenced by the dietary protein level in the growing chick and that the variations observed can be attributed to changes in the serum albumin level. It is of particular interest to note that the serum proteins and cholesterol can be altered by protein levels above that required for maximum growth. The changes observed in serum protein concentrations are reasonable when considered with respect to the concepts of a dynamic state of protein metabolism and of protein reserves.

As pointed out by Allison ('55), total serum protein or serum albumin is an indication of the protein reserves in an animal. The increase in serum albumin, and therefore total serum protein found when the dietary protein level was increased beyond the requirement for growth, reflects the ability of the chick to store "reserve" protein even after the animal has reached its maximum capacity for depositing tissue or less "labile" protein. The importance of such reserves in resisting the stresses of caloric restriction in the dog has been demonstrated by Rosenthal and Allison ('51). The data of Seeler and Ott ('45) demonstrate the importance of the protein reserves of the chick in resisting the stress of malarial infection. These authors infected chicks, fed various levels of protein, with malaria and noted that the chicks receiving the highest protein level were far more resistant to the infection.

The relative stability of the serum globulins as compared with the albumin level at the different levels of protein fed is in accord with observations in the rat (Allison, '55) and previous observations in the chick (Leveille et al., '60).

Reports have appeared indicating little or no change in serum protein level as a result of variations in dietary protein in the rat (Weimer et al., '59). In the protein-depleted pig, changes were observed (Peo et al., '57) during repletion in the various serum protein fractions; the globulin fractions were increased and the albumin fraction only slightly decreased. The resulting albumin globulin (A/G) ratios, however, were significantly decreased at the lower protein levels. Alterations in the A/G ratio have been reported as a consequence of a vitamin E deficiency in the chick (Goldstein and Scott, '56; Bieri and Pollard, 59) and various other pathological conditions in the chicken (Shelton and Olson, '60). The decreased A/G ratio usually observed in these pathological conditions is generally attributable to an increase in the serum globulin level. As pointed out by Allison ('55), such values are probably due to a loss of plasma water resulting in an apparent increase in the serum globulins. These changes in plasma volume are of utmost importance whenever expressing a plasma or serum protein component as units per volume; the basic assumption underlying such an expression, that of a constant plasma volume, is not always justified.

In the present study it has been shown that where plasma volume remained constant as a percentage of body weight, the only serum protein fraction which showed any variation due to dietary protein was the albumin fraction. Any deviation in plasma volume will, as mentioned above, introduce obvious errors; however, such variations are partially overcome by expressing the levels of albumin and globulin as A/G ratios as noted by Allison ('55).

The depression of serum cholesterol by high dietary protein levels is in accord with previous reports (Johnson et al., '58; Leveille and Fisher, '58; Leveille et al., '60). The mechanism by which such a depression is produced is not evident. It has been proposed that the observed effect of protein on serum cholesterol is secondary to the influence of dietary protein on liver function (Nishida et al., '60), the higher protein levels increasing the excretion of cholesterol in the form of bile acids. This hypothesis is supported by unpublished data from this laboratory, demonstrating a 45% increase in bile acid excretion (cholic and deoxycholic acids excreted per unit of body weight) by increasing the dietary protein level from 10 to 25%.

SUMMARY

Male chicks were fed protein levels of 10, 15, 20 and 25% for a three-week period. The influence of these variations

on growth, total serum protein components, serum cholesterol and on plasma volume was determined.

The protein requirement for maximum growth was found to be between the two highest protein levels fed and was calculated as 20.5% of the diet. Total serum protein increased from 2.33 gm per 100 ml at the 10% dietary protein level to 3.06 gm per 100 ml at the 25% level. The changes in serum protein could be attributed to variations in the albumin level, the level of the globulins remaining constant at all protein levels fed.

Serum cholesterol decreased from a level of 297 mg per 100 ml at the 10% protein level to 185 mg per 100 ml at the 25% level. Plasma volume remained constant as a percentage of body weight at the three highest protein levels fed.

The importance of variations in plasma volume on the determination of plasma components is discussed.

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Magnesium-28 Studies in Lambs[1]

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The importance of magnesium in metabolic functions throughout the animal body is reflected in the syndrome exhibited when blood plasma levels of this ion deviate appreciably from the normal. In ruminants, a physiological magnesium deficiency occurs in adult animals when turned onto fresh pasture during spring and autumn in certain areas of the world. These conditions are difficult to produce experimentally and the causative factor(s) are not well understood. Magnesium deficiency, however, can be readily induced within three to 4 weeks in three- to 6-month-old lambs fed a semipurified ration containing roughage with a low-magnesium content (McAleese and Forbes, '59).

Various workers (Aikawa et al., '58; Aikawa, '59, '60; Brandt et al., '58) have used Mg^{28} in rats, rabbits and humans in an effort to obtain more detailed knowledge of the function of this ion in metabolism. Only three reports were found on the use of this isotope in ruminant studies and in all three instances the results apply to a single adult wether supplied with adequate dietary magnesium. In this communication, the use of Mg^{28} given to lambs with experimentally produced magnesium deficiency and to those given adequate dietary magnesium is reported in an attempt to further evaluate the serious problem of hypomagnesemia in ruminants.

The objectives of the investigation were to: (1) discern the principal organs or sites of action of the magnesium ion in the animal body; (2) study the uptake, distribution and rate of clearance from the blood and bone of magnesium-deficient and of control lambs; and (3) investigate the absorption and excretion patterns of orally and of intravenously administered doses of Mg^{28}.

MATERIALS AND METHODS

The Mg^{28} was obtained from Brookhaven National Laboratory as magnesium chloride in concentrated hydrochloric acid. The specific activity as received was comparatively low (9 to 15 μc of Mg^{28} per 20 mg of stable magnesium), thus imposing limitations on the use of this isotope as a tracer. Another limitation is the short half-life of 21.3 hours.

Blood samples were taken from the jugular vein of the lambs before dosing to determine plasma magnesium and calcium, by the method of Malmstadt and Hadjiioannou ('59). After dosing, blood samples were taken at regular intervals in heparinized tubes for whole blood and plasma Mg^{28} determinations. Periodic collections of urine and feces were made until the end of the experimental period when the animals were sacrificed. The individual organs were weighed and samples taken for dry matter determination. The gamma emission from 3-gm samples of tissues and feces (wet weight) and 3-ml aliquots of whole blood, plasma and urine were each counted in a deep-well scintillation counter with a sodium-iodide crystal. The amounts of Mg^{28} in all samples and dosing solutions were corrected for physical decay and results expressed as percentage of dose.

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Oral doses were administered in gelatin capsules (containing the required amount of original stock Mg^{28} solution buffered with sodium acetate) using a balling gun. Intravenous dosing was by the nylon catheter procedure using 5% sodium acetate as wash liquid.

A total of 20 lambs was used in three experimental periods. Fall lambs were used in experiments 1 and 2 and spring lambs in experiment 3. To produce magnesium deficiency one group of lambs was maintained with a magnesium-deficient ration (McAleese and Forbes, '59) and a second group with the same ration supplemented with 600 ppm of magnesium as magnesium carbonate, $MgCO_3 \cdot Mg(OH)_2$. Initially the lambs were group-fed and given an ad libitum supply of food and distilled water, with corn cobs as the bedding material.

After the lambs had received the respective rations for three or 4 weeks, when the blood plasma magnesium of the deficient animals had fallen to approximately 1.0 mg per 100 ml, they were transferred to individual metabolism cages and acclimated for a few days before dosing with the isotope.

EXPERIMENTAL

Experiments 1 and 2. Experiment 1 was run as a pilot trial to determine the dosage level that would give sufficiently high tissue activity and blood counts for detection of the isotope over the experimental period. Four lambs were used in this experiment. Lambs 1 (magnesium-deficient) and 2 (control) were each orally dosed with 88 μc of Mg^{28} (120 mg of stable magnesium) and lambs 3 (magnesium-deficient) and 4 (control) were given 29 μc of Mg^{28} (40 mg of stable magnesium) intravenously. Blood samples were withdrawn at intervals after dosing and collections made of the total urine and feces excreted. The lambs were sacrificed 35 hours after dosing.

Experiment 2 was essentially a replication of experiment 1, except that the dosing solution used in experiment 2 had a lower specific activity. In this study, 30 μc of Mg^{28} (58 mg of stable magnesium) were given intravenously to a magnesium-deficient lamb and a control lamb, while another magnesium-deficient lamb and another control lamb were each given an oral dose of 91 μc of Mg^{28} (175 mg of stable magnesium).

The data from experiments 1 and 2 are in agreement and are discussed together.

Experiment 3. Experiments 1 and 2 demonstrated that tissue activity could be followed easily up to 35 hours with an intravenous dose of 30 μc of Mg^{28} to lambs weighing 60 to 70 pounds. At this dosage rate, however, 40 to 60 mg of stable magnesium was contained in the dose. This amount of magnesium was approximately one-third the amount of plasma magnesium in an average 60 to 70 pound lamb supplied with adequate dietary magnesium. Thus, administration of this quantity of isotope cannot be considered a tracer dose. In an attempt to overcome this handicap of low specific activity it was decided to give a dose of 10 μc of the isotope and sacrifice the animals at intervals after dosing.

Eight lambs were used in experiment 3 (i) and were divided into two groups. Lambs in group A were maintained with a magnesium-deficient diet and those in group B with the same diet supplemented with 600 ppm of magnesium as in experiments 1 and 2. When the plasma magnesium of the deficient lambs had fallen to less than 1 mg per 100 ml, all lambs were given 10 μc of Mg^{28} intravenously. A lamb from each group was sacrificed at the end of two, 4, 8 and 12 hours, respectively, after dosing. The various organs were removed, weighed and samples taken for radioactivity measurement and dry matter determination as reported previously.

Experiment 3 (ii) was designed to evaluate the gastrointestinal absorption and distribution in the body tissues and excretory pathways of an oral dose of Mg^{28} given to both magnesium-deficient and control lambs over a 60-hour experimental period.

Two lambs receiving the magnesium-deficient ration (17 and 18) and two receiving the magnesium-supplemented ration (19 and 20) were each given an oral dose of 100 μc of Mg^{28}. Feces and urine samples were collected at appropriate time intervals for assay of activity as in experiment 1. The lambs were sacrificed 60 hours after dosing to obtain various or-

gan weights and samples for radioactivity measurement and dry matter determination.

RESULTS AND DISCUSSION

Experiments 1 and 2

1. Blood and plasma. Plasma concentrations of calcium and magnesium are shown in table 1. The deficient lambs had low plasma magnesium values (0.6 to 0.9 mg per 100 ml), whereas the values for the controls fell within the normal range (2.3 to 2.5 mg per 100 ml). The lowered plasma calcium levels for the deficient lambs is a usual symptom of this condition in the ruminant. In figure 1 is shown the rate of clearance of Mg^{28} from the whole blood and plasma of both magnesium-deficient and control lambs after intravenous injection of the dose. The rate of disappearance of the isotope from the whole blood followed the same pattern as for plasma. Both plasma and whole blood curves for the deficient and control lambs took the same form but more Mg^{28} went from the blood of the deficient lambs to the tissues.

With oral dosing, the plasma Mg^{28} increased very sharply in the control lamb reaching a maximum 8 to 10 hours after dosing (fig. 2). The Mg^{28} concentration in the plasma of the deficient animal, however, did not increase as rapidly due pre-

TABLE 1

Magnesium and calcium in blood plasma (experiments 1 and 2)

Lamb no.	Deficient		Lamb no.	Control	
	Magnesium	Calcium		Magnesium	Calcium
	mg/100 ml			*mg/100 ml*	
5	0.8	7.9	4	2.3	10.7
3	0.9	8.7	6	2.5	10.2
1	0.7	9.0	2	2.4	9.7
7	0.6	7.3	8	2.5	10.3

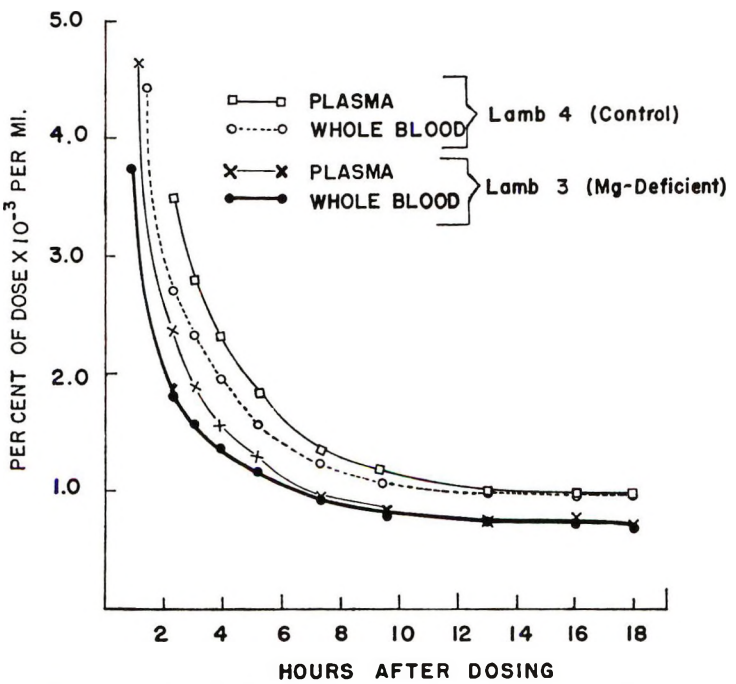

Fig. 1 Plasma and whole blood from lambs dosed intravenously with Mg^{28}.

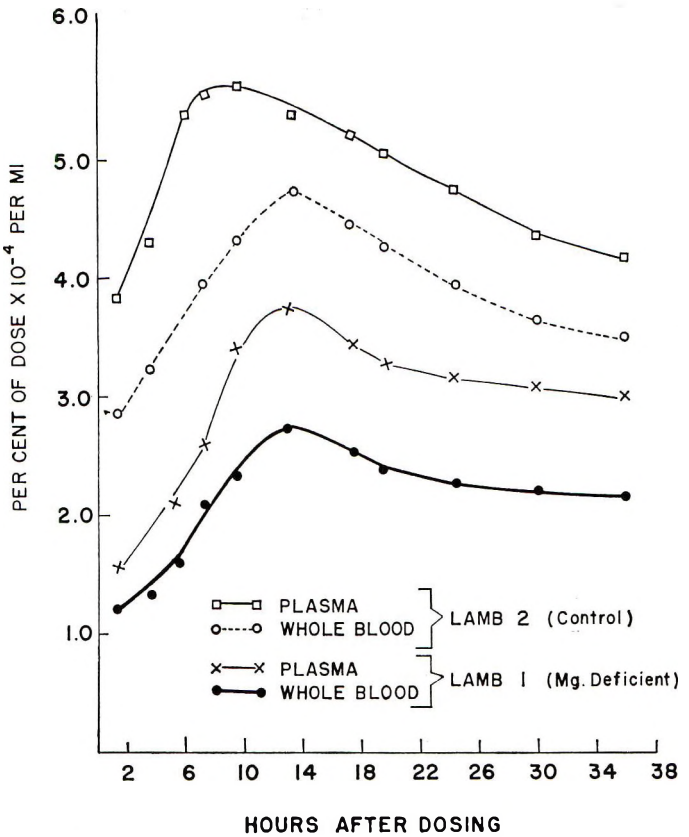

Fig. 2 Plasma and whole blood from lambs dosed orally with Mg^{28}.

sumably to a tissue deficiency, and reached a maximum about 12 to 16 hours after dosing. Mg^{28} content in the plasma then gradually declined throughout the remainder of the period of observation. The whole blood Mg^{28} picture followed the pattern of the corresponding plasma. The relatively constant displacement of the two curves indicates a very small uptake of Mg^{28} by the red blood cells either under deficiency or control conditions. The occurrence of smaller amounts of Mg^{28} in the blood of deficient animals was due to the equilibrium with deficient tissue since the percentage of dietary absorption was very much greater for this group.

The rapid rate of disappearance of Mg^{28} from the blood stream of control lambs is mainly due to urinary excretion although dilution in extracellular fluid and exchange by the various tissues also plays a part. For the magnesium-deficient lambs, the rapid rate of disappearance of plasma Mg^{28} is due principally to tissue uptake to replace lost magnesium rather than by exchange, the amounts excreted by way of urine being negligible under these conditions.

In these experiments the uptake of Mg^{28} by the red blood cells was very small. In fact, red cell activity was not statistically significant ($P > 0.05$) above that of the background, which supports the postulation that the lowered amounts of magnesium in red blood cells under magnesium deficiency conditions is due to a dilution by recently formed cells with lowered magnesium content rather than loss of magnesium from cells already formed. The negligible amounts of activity found in the bone marrow support this postulation.

2. *Excretion.* The percentage of the dose of Mg^{28} excreted in the feces after intravenous injection was small (3.6 to

TABLE 2

Fecal and urinary excretion of Mg^{28} over 35-hour experimental period (experiments 1 and 2)

Dose	Lamb no.	Deficient		Lamb no.	Control	
		Feces	Urine		Feces	Urine
		%	%		%	%
Intravenous	5	2.1	< 0.1	4	3.6	7.2
Intravenous	3	1.7	< 0.1	6	5.0	5.9
Oral	1	23.3	1.0	2	58.2	7.8
Oral	7	30.9	0.8	8	65.9	4.4

5.0%) for control lambs but higher than for the deficient animals (1.7 to 2.1%) over the 35-hour experimental period (table 2). The controls, however, had a much greater urinary output (5.9 to 7.2%) compared with that (< 0.1%) eliminated by this route in the deficient lambs. With oral dosing the urinary picture was similar to that for intravenous administration; namely, larger amounts of the isotope occurred in the urine of the control lambs but 58 to 66% of the given dose was found in the feces of the control lambs.

Although the data reported in the literature on the intestinal absorption of magnesium are rather variable, Field ('59) and MacDonald et al., ('59) obtained comparable figures for the absorption of carrier free Mg^{28} using a single adult wether in each case.

3. Soft tissues. The soft tissues from the deficient lambs had higher concentrations of Mg^{28} than did the corresponding tissues from the control lambs (table 3).

The concentration of Mg^{28} was found to be greatest in the heart, decreasing in the order of liver, spleen and kidney. In terms of total uptake the liver contained the largest amount of Mg^{28}. The occurrence of considerable amounts of this isotope in these organs would be expected since they are sites of much enzymatic activity. Although much of the high activity occurring in the kidneys of control lambs, especially after intravenous dosing, is due to the fact that this is the principal excretory pathway of excess magnesium, the presence of appreciable amounts in the kidneys of the deficient lambs points to a role of this ion in kidney function. The uptake of Mg^{28}

TABLE 3

Mg^{28} uptake by tissues, teeth and bone of lambs 35 hours after dosing

Lamb no.	Intravenous dose				Oral dose			
	Deficient		Control		Deficient		Control	
	5	3	4	6	1	7	2	8
Heart	1.159[1]	1.360	0.655	0.756	0.467	0.505	0.309	0.219
	0.048[2]	0.051	0.019	0.023	0.016	0.018	0.007	0.008
Liver	3.314[1]	3.388	1.313	1.019	1.106	1.129	1.194	0.787
	0.033[2]	0.029	0.011	0.010	0.010	0.013	0.007	0.007
Spleen	0.104[1]	0.121	0.060	0.074	0.073	0.099	0.053	0.032
	0.013[2]	0.014	0.008	0.009	0.008	0.006	0.004	0.004
Lung	0.746[1]	0.960	0.624	0.481	0.234	0.228	0.157	0.166
	0.009[2]	0.012	0.007	0.008	0.004	0.003	0.002	0.003
Kidney	0.126[1]	0.115	0.090	0.082	0.074	0.055	0.041	0.048
	0.014[2]	0.014	0.010	0.010	0.009	0.007	0.004	0.005
Brain	0.053[1]	0.070	0.017	0.012	0.035	0.037	0.022	0.019
	0.003[2]	0.004	0.001	0.001	0.002	0.002	0.001	0.001
Muscle	0.003[2]	0.003	0.001	0.001	0.001	0.001	0.001	0.001
Teeth	0.011[2]	0.009	0.006	0.005	0.004	0.005	0.002	0.002
Bone shank	0.007[2]	0.006	0.015	0.014	0.001	0.002	0.006	0.005
Bone epiphysis	0.010[2]	0.007	0.009	0.008	0.004	0.003	0.001	0.001
Bone marrow	0.001[2]	0.001	0.001	0.001	0.001	0.001	0.001	0.001

[1] Percentage of dose per total organ.
[2] Percentage of dose per gram of dry matter.

by the lungs of all the experimental animals is difficult to interpret although this organ is acknowledged to have much enzymatic activity. The greater accumulation of Mg^{28} by the lungs of the deficient lambs precludes the accidental presence of this ion in the alveolar tissue.

4. Bone and teeth. Control lambs had approximately twice as much Mg^{28} in the diaphysis portion of the long bones as did the deficient lambs; however, the activity occurring in the epiphyseal region of the long bones was the same for each group and of the same concentration as found for the diaphysis section of the long bones from the control lambs. The amount of penetration of Mg^{28} into the bone marrow was negligible for all experimental animals. Variation in uptake of Mg^{28} among areas of bone has been pointed out by Field ('60).

The rate of Mg^{28} uptake by soft tissues of both control and deficient lambs was much greater than that for bone. This supports the conclusion of Glaser et al. ('58) for uptake of Mg^{28} in man and dogs, but Aikawa ('60) and Aikawa and Burns ('60) found that in the rabbit, Mg^{28} is absorbed more rapidly by bone than by any other tissue. Since approximately 70 to 75% of total body magnesium occurs in bone, of which 80 to 90% has been shown to be labile (McAleese and Forbes, '61), uptake of Mg^{28} by bone from control lambs by simple exchange should be greater than that for the tissues. Results presented herein, however, do not support this postulation and on this premise it may be deduced that the magnesium reserve in bone is mobilized under dietary magnesium inadequacy. Two theories may be advanced to account for the greater uptake of Mg^{28} by the long bones of the animals in the control groups compared with the uptake by the long bones of the deficient lambs. Most of the reports in the literature (Watchorn and McCance, '37; Duckworth and Godden, '41, '43; Smith, '59) confirm the reserve character of much of the magnesium occurring in bone. Therefore, when the animal is supplied with a restricted amount of additional magnesium after a period of deficiency, the bone is unable to compete for the additional supply of magnesium until the soft tissues have obtained their needs. The magnesium released from bone during depletion period is replaced by an equivalent amount of calcium (McAleese and Forbes, '61). During the repletion period the magnesium ions displace this calcium and this is a relatively difficult and slow process since at pH7 the magnesium ions exist as $Mg(OH)^{+}$. The slow uptake of Mg^{28} by bone on this basis is therefore due to mechanical blocking, the bone, in fact, having equal competitive power to that of other tissues. Probably, both theories are operative which would explain the variable results obtained by workers in this field.

Mg^{28} was concentrated in teeth to a remarkable degree in the deficient lambs, accumulating twice the amount as in the teeth from control lambs within two hours after dosing. This is unlike the situation existing for bone and is not readily explained.

Experiment 3

1. Plasma. The amounts of calcium and magnesium in the plasma, just before administration of the dose, of all lambs used in experiment 3 are given in table 4.

TABLE 4

Magnesium and calcium in the blood plasma of lambs (experiment 3)

Lamb no.	Deficient		Lamb no.	Control	
	Magnesium	Calcium		Magnesium	Calcium
	mg/100 ml			*mg/100 ml*	
9	0.5	8.6	13	2.2	11.2
10	0.6	11.1	14	2.0	11.9
11	0.3	8.4	15	2.0	10.3
12	0.7	10.5	16	2.2	10.9
17	0.5	8.6	19	2.5	11.6
18	0.6	10.0	20	2.1	11.5

The deficient lambs had 0.7 mg per 100 ml or less magnesium in the plasma, whereas the values for the control lambs range from 2.0 to 2.5 mg per 100 ml. These data are in agreement with data obtained in experiments 1 and 2.

2. *Tissue.* Distribution of Mg^{28} in the tissues of the 8 animals in experiment 3 after intravenous dosage, is shown in table 5 and after oral dosage, in table 6. The content of Mg^{28} in the soft tissues of deficient animals was at least twice and for some organs three to 4 times that of the tissues from the control lambs even within two hours after dosing.

The highest concentration of Mg^{28} both for deficient and control groups occurred in the kidney, heart, liver, spleen and lungs in that order. Although much of the higher concentration occurring in the kidney may be attributed to this being the main excretory pathway for the magnesium ion, the presence of this ion in appreciable amounts in the kidney of the deficient animal, where urinary excretion of Mg^{28} is negligible, suggests a role for this ion in the kidney. MacIntyre and Davidson ('58) showed a reduction in magnesium deposition and a 4-fold increase in calcium content of kidney from rats fed magnesium-deficient diets. In the control lambs maximum concentration of Mg^{28} occurred in the tissues approximately 8 hours after intravenous dosing corresponding to the period when the blood level had reached a low concentration. Plasma clearance of Mg^{28} appears to be largely due to tissue uptake. Generally the greatest uptake of Mg^{28} by tissues of the deficient animals occurred at the same time or earlier than that by the controls. Although the concentration in the skeletal muscle was small, the amount of Mg^{28} in muscle at any given time would account for a considerable portion of the administered dose, due to the large amount of muscle. Smaller amounts of Mg^{28} occurred in the brain of controls than in the brain of deficient lambs. In each case the concentrations in muscle and brain dry matter were similar.

In terms of total Mg^{28} uptake by total organs, the liver was observed to have the greatest amount of the isotope followed by the lung, heart, kidney and spleen.

As noted in experiments 1 and 2, more Mg^{28} was observed in the diaphysis portion of the long bone from the control lambs compared with the deficient animals, although similar amounts occurred in the epiphyseal area from both groups. Only negligible amounts of Mg^{28} could be detected in the bone marrow. Although teeth from the deficient lambs had larger uptake of Mg^{28}, the considerable amounts occurring in the teeth of the controls shows the avidity of teeth for this ion and the dynamic character of teeth at least during the growth period.

The tissue distribution of Mg^{28} from the orally dosed lambs of experiment 3 is in agreement with intravenous dosing—the highest concentration occurred in the tissues from the deficient lambs although the actual amounts were much smaller in the case of oral dosing. Liver accumulated the greatest amount of Mg^{28}, followed in order of amount by the lung, heart, spleen and kidney. The distribution in the kidneys from the oral dosing was much less than when the dose was given intravenously.

The teeth from deficient lambs had 4 times the Mg^{28} concentration as compared with controls. The amounts in the shank portion of the long bones, however, were the same for both groups in contrast with the findings for intravenous dosing.

3. *Urine and feces.* The percentages of the dose (oral) occurring in the feces and urine over the 60-hour experimental period for the 4 lambs of experiment 3 are given in table 7. The two lambs fed supplementary magnesium excreted 52 and 50%, respectively, of the radioactive dose in the feces. These amounts contrast with the low values of 25 and 30% excreted in the feces by the two deficient lambs. Excretion of the isotope in the feces reached a maximum 14 to 38 hours after dosing for the control lambs and 33 to 43 hours after dosing for the deficient lambs. Approximately 20% of the dose occurred in the urine of the control lambs, and less than 1% of the dose was eliminated via this route by the deficient lambs. These results show the apparent gastrointestinal absorption of Mg^{28} to be at least two times greater in the deficient lambs which is re-

TABLE 5

Distribution of Mg^{28} in tissues at various intervals after intravenous dose

Hours after dosing	Heart		Liver		Spleen		Lung		Kidney		Brain	
	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control
2	4.019[1]	1.323	10.187	3.751	0.590	0.207	4.695	1.595	1.133	0.680	0.177	0.013
	0.093[2]	0.030	0.072	0.022	0.044	0.018	0.047	0.016	0.125	0.052	0.013	0.001
4	3.087[1]	1.540	6.751	4.780	0.751	0.277	2.524	2.260	0.816	0.516	0.081	0.026
	0.095[2]	0.043	0.060	0.033	0.056	0.025	0.029	0.025	0.097	0.065	0.004	0.002
8	3.593[1]	2.051	10.262	5.386	0.600	0.371	2.580	2.208	0.972	0.488	0.209	0.120
	0.106[2]	0.060	0.085	0.040	0.065	0.036	0.034	0.032	0.102	0.060	0.006	0.007
12	3.209[1]	1.634	7.330	4.531	0.643	0.346	2.745	1.961	0.590	0.460	0.295	0.140
	0.099[2]	0.027	0.060	0.024	0.050	0.024	0.039	0.020	0.072	0.040	0.017	0.007

Hours after dosing	Muscle		Teeth		Bone shank		Bone epiphysis		Bone marrow	
	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control
2	0.006[2]	0.003	0.005	0.002	0.004	0.008	0.007	0.006	0.001	0.001
4	0.004[2]	0.004	0.009	0.002	0.008	0.015	0.012	0.011	0.002	0.001
8	0.006[2]	0.006	0.012	0.007	0.008	0.014	0.015	0.015	0.002	0.002
12	0.006[2]	0.003	0.010	0.007	0.008	0.018	0.010	0.010	0.001	0.002

[1] Percentage of dose per total organ.
[2] Percentage of dose per gram of dry tissue.

TABLE 6

Distribution of Mg^{28} in tissues 60 hours after oral dosing

Ration	Lamb no.	Heart	Liver	Spleen	Lung	Kidney	Brain	Muscle	Teeth	Bone shank	Bone epiphysis	Bone marrow
Deficient	18	0.775[1]	2.180	0.113	1.142	0.167	0.120					
		0.021[2]	0.018	0.012	0.013	0.017	0.007	0.007	0.012	0.005	0.005	0.001
	17	0.822[1]	2.969	0.218	1.158	0.225	0.117					
		0.021[2]	0.018	0.020	0.014	0.020	0.005	0.007	0.012	0.004	0.004	0.001
Control	19	0.282[1]	0.828	0.110	0.607	0.082	0.070					
		0.009[2]	0.006	0.010	0.007	0.009	0.005	0.004	0.004	0.004	0.003	0.001
	20	0.391[1]	1.047	0.129	0.531	0.074	0.049					
		0.010[2]	0.007	0.009	0.007	0.008	0.004	0.002	0.004	0.005	0.005	0.001

[1] Percentage of dose per total organ.
[2] Percentage of dose per gram of dry tissue.

TABLE 7

Fecal and urinary excretion of Mg^{28} over 60-hour period after oral dosing

Ration	Lamb no.	Collection period (hours)							Total
		0–10	10–14	14–19	19–33	33–38	38–43	43–60	
					% of dose				%
Deficient	17	0.01[1]	0.04	0.19	11.86	8.63	6.77	2.48	29.98
		Counts not significant[2]							< 0.1
	18	0.05[1]	1.60	3.67	6.90	6.64	4.96	0.75	24.57
		Counts not significant[2]							< 0.1
Control	20	2.74[1]	10.35	13.30	18.99	2.30	0.53	3.36	51.57
		4.49[2]	4.32	2.85	4.01	1.31	1.07	3.18	21.83
	19	0.04[1]	0.36	1.36	26.43	13.05	4.78	4.10	50.12
		3.37[2]	2.71	—	9.23	2.09	0.50	2.07	19.97

[1] Feces.
[2] Urine.

flected by the larger amounts occurring in the tissues of the deficient group.

Daily ad libitum feed consumption for the control lambs was about 900 gm and the intake of the deficient animals was of the order of 400 gm. This was reflected in the larger fecal output of the controls (2287 and 1599 gm) compared with the total fecal output of 1199 and 900 gm for the deficient lambs. Total amounts of urine eliminated were 2916 and 2942 ml, by the deficient lambs and 1877 and 2333 ml by the control lambs. Aikawa ('59) showed that restricting feed intake in rabbits reduced the fecal excretion of Mg^{28} but had no effect on the urinary excretion of Mg^{28}.

Data reported herein for three- to 5-month-old lambs supplied with adequate dietary magnesium show that the apparent absorption of Mg^{28} from the gut ranges from 40 to 50% with a net retention of 16 to 30%. Using magnesium-deficient lambs, however, the apparent absorption of a given dose ranges from 70 to 75%, which amount is retained since only trace amounts occur in the urine under these conditions. Although these experiments extended over a 60-hour period and prolongation may have given rise to the excretion of additional amounts of isotope, these amounts would not be expected to have been sufficiently great to alter the results obtained. Obviously, magnesium status of the animal and age (Field, '59; MacDonald et al., '59) have an important bearing on the efficiency of magnesium absorption. The high percentage of absorption and retention by the magnesium-deficient lambs reflect the adaptive powers of the body to meet dietary needs.

During the course of these experiments, two lambs not included in the experiments described but fed magnesium-deficient ration, died showing acute symptoms of magnesium tetany.

SUMMARY

Magnesium-deficient and control lambs were dosed both orally and intravenously with Mg^{28}. Distribution of the isotope in the various tissues was measured in addition to the rate of disappearance of Mg^{28} from the plasma and whole blood. The excretory pathways were also investigated in these studies.

1. Plasma disappearance of intravenously administered Mg^{28} was very rapid during the first two hours after dosing followed subsequently by a slower exponential disappearance until approximately 8 to 10 hours when most of the blood activity had disappeared.

2. Uptake of this isotope by the red blood cells was very small even for the deficient lambs. The shape of the Mg^{28} plasma and whole blood disappearance curves was the same for both the control and deficient lambs with smaller amounts of Mg^{28} occurring in the blood of the deficient group.

3. Plasma Mg^{28} reached a maximum 12 to 14 hours after oral dosing. The amounts of isotope in the blood of the deficient lambs were less than that of controls due to the greater uptake by the tissues from the deficient group.

4. Tissues from the deficient animals had higher concentration and total amounts of the isotope as compared with control lambs. Although the heart, liver, spleen, lung and kidney were the most active sites of Mg^{28} activity, the ubiquitous character of this ion in the animal body was shown by its widespread distribution in other tissues.

5. Although a higher concentration of Mg^{28} was found in the teeth of the deficient lambs than of controls, the amounts occurring in the latter show the remarkable avidity of this tissue for magnesium. These results indicate the dynamic character of teeth in young animals and a possible requirement for magnesium in normal tooth development.

6. The long bones from the control group, particularly the shank portion, contained the highest isotope activity. Only negligible amounts were observed in the bone marrow of either deficient or control lambs.

7. Absorption of magnesium was much greater, and urinary excretion was much less, by deficient lambs than by control lambs.

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INVITATIONS FOR NOMINATIONS FOR 1962 AMERICAN INSTITUTE OF NUTRITION AWARDS AND FELLOWS

Nominations are now being invited for the 1962 A. I. N. awards and Fellowships.

Nominations for the 1962 *Borden Award in Nutrition* must be submitted by October 1, 1961, to Dr. E. E. Snell, Department of Biochemistry, University of California, Berkeley 4, California.

Nominations for the 1962 *Osborne and Mendel Award* are due also by October 1, 1961, and should be sent to Dr. Grace A. Goldsmith, Department of Medicine, Tulane University School of Medicine, New Orleans 12, Louisiana.

The deadline for receipt of nominations for *A. I. N. Fellows* is October 1, 1961. These should be sent to Dr. E. W. McHenry, School of Hygiene, University of Toronto, Toronto, Ontario, Canada.

Full details of the rules for these awards and lists of former recipients are given in the July 1961 issue of the *Journal of Nutrition*.

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2. Name or names of authors.
3. Institution or laboratory, with city and state.
4. Shortened form of title (not more than 48 letters and spaces).
5. Address and name of author to whom proof is to be sent.

(Place footnotes on a separate sheet, typed double-spaced.)

The text should begin on page 2. The manuscript should be submitted in complete and finished form exactly as the author wishes it to appear in type. Citations of the literature in the text should be made by author and year of publication, as (Jones, '58). The words et al. are not underscored, nor is ad libitum.

Never divide a word at the end of a line, for, it might be set incorrectly with a hyphen. The repeated use of a unit in a sentence may be avoided by stating the common unit used as, "the diet consisted of the following in milligrams: sucrose, 6; etc." Or, in other cases, as "8, 12 and 14%."

A common error occurs in describing the percentage of a compound. Use the form, "15% of calcium" when the percentage is not an adjective. Number all pages consecutively in the following order: title page, text, tables, figures and legends, literature cited and footnotes.

Start each of the following sections on a new page and typed double-spaced:

1. Tables, with appropriate heading (a separate sheet for each table)
2. Explanation of figures, numbered and listed consecutively
3. Literature Cited, arranged alphabetically by authors—see current issue for exact form, and
4. Footnotes.

Footnotes. In the text, footnotes should be numbered consecutively, including any indicated on page 1. In a table, footnotes should be made part of the table, typed directly beneath it, and numbered 1, 2, 3, etc. These are independent of other footnote numbers used in the manuscript.

Acknowledgments. Financial support should be listed as a footnote to the title. Credit for materials should be listed as a footnote in the text. Technical assistance and advice may be cited in a section headed Acknowledgments, which will follow the summary.

Summary. This should represent a brief recapitulation of the problem and results.

Literature Cited. Accuracy and adequacy of the references are the responsibility of the author. Literature cited should be checked carefully with the original publication. References to abstracts of verbal reports, personal communications or unedited reports should be treated as text footnotes. Reference to a paper in press may be included in Literature Cited. If such a paper would be useful in the evalua-

tion of the manuscript under consideration, it is advisable to make a copy of it available to the Editor. When a manuscript is one of a series of papers, the preceding paper should be included in Literature Cited.

Numbers. If possible, avoid using a number as the first word in a sentence. Numbers so used should always be spelled out, but no abbreviations should follow such numbers. Numbers indicating time, weight and measurements are to be expressed in Arabic figures when followed by an abbreviation (e.g., 2 mm; 1 sec.; 3 gm; 100 mm^3). Except as indicated, the numbers *one*, *two* and *three* will be spelled out in the text. All higher numbers will be given in Arabic figures. Use Arabic numerals throughout, including tables.

Abbreviations. The word *figure*, written in the text, is not abbreviated except when appearing in parentheses, as (fig. 2) (figs. 4–6).

The words *pounds*, *weeks* and *days* are not abbreviated.

The metric system should be used in preference to others for measurements, weights, etc. Temperatures should always be expressed in Centigrade scale. Metric abbreviations listed below are shown in the approved lower-case style, *without periods:*

	Weight		*Length*
kg	kilogram	km	kilometer
gm	gram	m	meter
mg	milligram	cm	centimeter
μg	microgram	mm	millimeter
mμg	millimicrogram	μ	micron
μμg	micromicrogram	mμ	millimicron
		μμ	micromicron
	Volume		
m^3	cubic meter		
cm^3	cubic centimeter		*Area*
mm^3	cubic millimeter	m^2	square meter
l	liter	cm^2	square centimeter
ml	milliliter	mm^2	square millimeter

Symbols. When preceded by a figure, the following symbols are used:

A	angstrom units	°	degree
%	per cent	ppm	parts per million

Tables and figures. Follow form in current issues for the use of upper and lower case letters and italics. Authors are urged to economize on space used for tables and figures. These should fit one column width (2⅝ inches) or when necessary, two column widths (5½ inches). A charge will be made for that space used for tables and figures which exceeds one-half of the space used for the manuscript exclusive of tables and figures. A table or figure should be constructed so as to be intelligible without reference to the text. Lengthy tabulation of essentially similar data can often be avoided by giving the number of experimental results and their mean values, with standard deviations or ranges within which the values fall. Statements that significant differences exist between the mean values of two groups of data should be accompanied by indications of probability derived from the test of significance applied.

Original drawings, with two reproductions, to be sent to reviewers, or in the case of photographs, the original and two glossy prints, should accompany the manuscript. They should be marked on the back in ink with the author's name and complete address. Such drawings and photographs must not exceed 8½ × 11 inches in size and must be at least 5¼ inches wide in order to fit the 2⅝-inch single column width when reduced by one-half. When a complicated figure requires more space for clarity, a proportionately larger illustration will be acceptable. Drawings should be on white or blue-white paper or bristol board—not cream-white. They should be prepared for reproduction as line or halftone engravings. Letters and numbers should be uniform and large enough so that no character will be less than 2 mm high after reduction. A line of 0.4 mm wide reproduces satisfactorily when reduced by one-half.

Figures should be lettered in *black* India ink. For any charts made on cross-section paper, use India ink on paper printed in light blue only. Charts drawn in India ink should be so executed throughout, with no typewritten material included. Graphs and charts should be given consecutive figure numbers as they will appear in the text.

American Documentation Institute. To conserve space in the Journal, lengthy descriptions of experimental procedures, detailed data such as that on individual animals or subjects, extra figures and other material considered important supplementary information, may be deposited with the American Documentation Institute, Library of Congress, through the Editor's office with a footnote reference.

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ERRATUM

Lease, J. G., B. D. Barnett, E. J. Lease and D. E. Turk 1960 The biological unavailability to the chick of zinc in a sesame meal ration. J. Nutrition, 72: 66.

(Ms. error) On page 67, column 2, the sentence beginning on line 6 should have read as follows:

The L-lysine content of the 1957 sesame meal was 23 mg per gm; that of the 1958 sesame meal 19 mg per gm on the fat-free basis.

The entire paragraph from top of second column, page 67 is reprinted here for cutting and pasting over the incorrect original printing.

> sesame meal, 52 ppm; soybean oil meal, 29 ppm; and isolated soybean protein, 12 ppm. The zinc content of the sesame meal and soybean oil meal rations in experiment 1 were similar to those in experiment 2. The L-lysine content of the 1957 sesame meal was 23 mg per gm; that of the 1958 sesame meal 19 mg per gm on the fat-free basis. The L-lysine of the sesame meal in the ration plus that of the added L-lysine hydrochloride totaled 1.4% of L-lysine in the sesame meal rations.