Hepatic Nucleotide Levels and NAD-synthesis as Influenced by Dietary Orotic Acid and Adenine

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ABSTRACT A simplified method is described for quantitating the effect of dietary orotic acid on the hepatic concentrations of adenine, guanine and uracil nucleotides in the rat. Within 16 hours after addition of 1% orotic acid to the diet, hepatic uracil nucleotide concentrations are 4 times normal, and concentrations of acid-soluble adenine, guanine and oxidized pyridine nucleotides have begun to decrease, coincident with a previously reported decrease in plasma lipid concentrations. Minimum values are observed after about 4 days. These effects of orotic acid are all rapidly reversed when 0.25% adenine sulfate is added to the diet. Addition of nicotinamide or nicotinic acid restores to normal only the depressed pyridine nucleotide concentration. Nicotinamide and nicotinic acid mononucleotide administered parenterally induce a 2- to 2.5-fold increase in hepatic oxidized pyridine nucleotide concentration of normal rats, but they have only slight effects in rats fed 1% orotic acid for 72 hours. The efficacy of nicotinamide in orotic acid-fed rats is improved by the administration of adenine. Depressed synthesis of pyridine nucleotides in rats fed orotic acid appears to reflect a relative unavailability of acid-soluble adenine nucleotides due either to an inhibition of purine synthesis *de novo* or increased competition for adenine by other metabolic pathways.

Feeding high concentrations of orotic acid to rats produces a massive accumulation of hepatic lipids, mostly triglyceride (1, 2). This apparently results from an inhibition of lipoprotein secretion by this organ (3,4). The fatty liver is refractory to the administration of choline, methionine or other so-called lipotropic agents (1), but is prevented completely and also corrected by the supplementation of the diets with adenine (5).

Feeding 1% orotic acid has also been reported to result in changes in concentrations of certain acid-soluble nucleotides of liver. Two- to fourfold increases in uracil nucleotides accompanied by a depression of adenine and pyridine nucleotides have been observed (6,7). Adenine and uracil nucleotides were determined in these studies by the use of ion-exchange chromatography.

The present report describes a simplified procedure, easily applied to large numbers of animals, for following hepatic nucleotide concentration changes induced by orotic acid. The procedure has been used to study the time-course of these changes as well as the onset and extent of their reversal with adenine. Also, the synthesis of pyridine nucleotides from various precursors in the orotic acid-fed rat was studied.

EXPERIMENTAL PROCEDURES

Animals and diets. Unless otherwise noted, male rats of the Sprague-Dawley strain weighing 40 to 120 g were caged individually in wire-bottom cages and provided with food and water ad libitum. All groups were fed a basal diet for an adjustment period of 7 to 10 days before experimental supplements were tested. Basal diet W-4 is a complete purified mixture as described by Handschumacher et al. (5)[1] with casein increased to 22%

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[1] Diet W-4 contained the following: (in g/kg of diet) casein (vitamin-free, Nutritional Biochemicals Corp., Cleveland), 220; sucrose, 685; corn oil, 20; vitamin diet fortification mixture (Nutritional Biochemicals Corp.), 22; HMW salt mixture, 50; and L-cystine, 3.

The vitamin mixture, triturated in dextrose, provided the following: (in mg/kg of diet) vitamin A concentrate, 200 units/mg, 99; vitamin D concentrate, 400 units/mg, 5.5; α-tocopherol, 110; ascorbic acid, 990; inositol, 110; choline chloride, 1650; riboflavin, 22; menadione, 49.5; *p*-aminobenzoic acid, 110; niacin, 99; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; biotin, 0.44; folic acid, 1.98; and vitamin B_{12}, 0.297.

The HMW salt mixture had the following percentage composition: $CaCO_3$, 54.300; $MgCO_3$, 2.500; $MgSO_4 \cdot 7H_2O$, 1.600; NaCl, 6.900; KCl, 11.200; KH_2PO_4, 21.200; $FePO_4$, 2.050; KI, 0.008; $MnSO_4 \cdot H_2O$, 0.035; NaF, 0.010; $AlK(SO_4)_2 \cdot 12H_2O$, 0.017; and $CuSO_4 \cdot 5H_2O$, 0.090.

plus the addition of 0.3% L-cystine. Basal diet R1FF is a fat-free but otherwise complete purified mixture and has been previously described (4).[2] All injections were via the intraperitoneal route in 0.5 to 1.0 ml of 0.95% sodium chloride.

Preparation of liver extracts. Rats were decapitated and within 45 seconds a piece of liver was excised and frozen in liquid nitrogen. A weighed portion (about 1 g) was homogenized without previous thawing in 9 ml of ice-cold 5% perchloric acid. A portion of the supernatant fraction obtained by centrifugation was adjusted to pH 6 to 7 with KOH, and potassium perchlorate was removed by centrifugation. The neutralized extracts were stored at −25° until analysis was performed.

Determination of acid-soluble components. The adenine and guanine in the neutralized extracts were isolated by a modification of the silver precipitation procedure of Kerr and Seraidarian (8). A 4-ml portion of each extract was acidified by addition of H_2SO_4 to a final concentration of 0.1 N, then heated in a boiling water bath for 2 hours. After cooling, 5 N NaOH was added to a final pH of 1. The extracts were warmed to 80° and 1 ml of 1 M $AgNO_3$ was added. Insoluble silver salts were allowed to precipitate at 4° overnight. After centrifugation, the clear supernatant fraction was decanted and used for uridine analysis, as described below. The precipitated silver salts were washed 3 times on the centrifuge with 4 ml of an ice-cold solution of 0.05 M $AgNO_3$ in 0.1 N H_2SO_4. The washings were discarded. Purines were extracted by suspending the washed silver precipitate in 4 ml of 0.5 N HCl, heating in a boiling water bath for 15 minutes, cooling, and centrifuging. An aliquot of the clear, colorless supernatant solution was diluted with 6 volumes of water and its absorbancy determined at 240, 252, 262, 276 and 280 mμ. From the values obtained, the adenine and guanine concentrations were determined using the formulas developed by Loring et al. (9). The validity of the method depends upon the complete precipitation by silver ions of only adenine and guanine. Kerr and Seraidarian (8) have provided evidence for the completeness of precipitation. When the HCl extracts of the silver precipitates of several livers were concentrated and submitted to paper chromatography (descending), using Whatman no. 1 paper, only 2 ultraviolet-quenching spots were observed, and their mobilities corresponded to those of adenine ($R_f = 0.37$) and guanine ($R_f = 0.14$). The developing solvent was water-saturated *n*-butanol with 1% (v/v) 15 N NH_4OH (10).

Total oxidized pyridine nucleotides (nicotinamide adenine dinucleotide (NAD) + nicotinamide adenine dinucleotide phosphate NADP)) were assayed in 50 μliters of the perchloric acid extracts (before neutralization) by the fluorometric procedure of Lowry et al. (11). For each sample a blank was prepared in which pyridine nucleotides were destroyed by heating for 2.5 minutes in the presence of 0.09 M K_2CO_3. The fluorescence of each sample was corrected by the value for the corresponding blank.

The increase in concentration of uridine nucleotides which resulted from orotic acid feeding was quantitated by measuring the difference in absorbancy at 262 mμ (pH 1) of purine-free acid extracts (the supernatant solutions after removal of insoluble silver salts) of livers of orotic acid-fed rats and appropriate control rats. The validation of this procedure will be discussed in the Results section. In the calculations, the molar extinction coefficient of uridine was taken as 10.1×10^3 (12). The absolute concentration of uridine nucleotides was obtained by adding the increase due to the orotic acid feeding to the amount normally found in control rats under similar conditions, namely, 2 μmoles /g of liver (7).

[2] Diet R1FF contained the following: (in g/kg of diet) glucose monohydrate, 689.8; casein (General Biochemicals, Inc., Chagrin Falls, Ohio), 200; vitamin mixture, 45; salt mixture, 60; DL-methionine, 3; choline chloride, 2; and *i*-inositol, 0.2.

The vitamin mixture, made up in glucose monohydrate, provided the following: (in mg/kg of diet) thiamine·HCl, 8; riboflavin, 8; Ca pantothenate, 20; niacin, 100; pyridoxine·HCl, 8; D-biotin, 0.3; folic acid, 3; and vitamin B_{12}, 0.02. Fat-soluble vitamins were given separately to each rat in 2 drops of corn oil each week so that each rat received 0.97 mg of vitamin A acetate, 0.0007 mg of vitamin D_3, 4.9 mg of *dl*-α-tocopheryl acetate, and 0.42 mg of 2-methyl-1,4-naphthoquinone per week.

The salt mixture provided the following: (in g/kg of diet) $CaHPO_4$, 28.4; $CaCO_3$, 10; NaCl, 4; Na_2HPO_4, 7; KCl, 7; $MgSO_4$ (anhydrous), 3; ferric citrate, 0.2; $MnSO_4 \cdot H_2O$, 0.25; KIO_3, 0.01; $ZnCO_3$, 0.13; $CuSO_4$ (anhydrous), 0.01; and $Na_2MoO_4 \cdot 2H_2O$, 0.005.

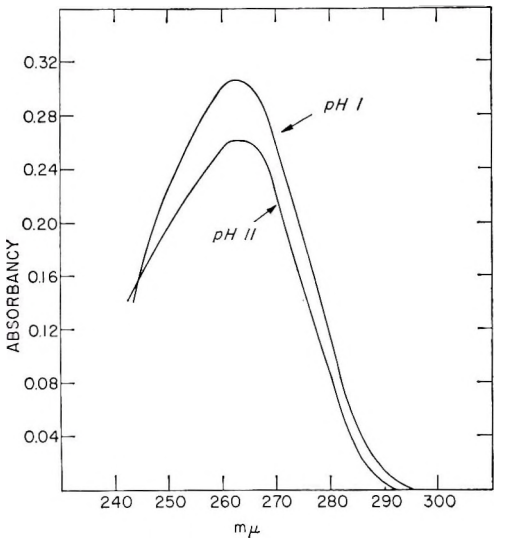

Fig. 1 Typical difference spectra of a purine-free acid extract of liver from an orotic acid-fed versus a similar extract from a control rat. One-gram samples of liver were extracted with perchloric acid and, following hydrolysis, the purines were precipitated with $AgNO_3$, as described under Experimental Procedures. A portion (0.3 ml) of the purine-free extract was diluted to 3.0 ml with 0.1 N H_2SO_4 (pH 1) or 1 N NH_4OH (pH 11). The absorbancy of the extract from a rat fed 1% orotic acid for 4 days was recorded, using as a blank a similar extract from a rat fed only the basal diet (W-4).

Liver lipid. The total lipid concentration of livers was determined as described previously (3).

Materials. Nicotinamide mononucleotide was obtained commercially.[3] From this, NaMN was prepared by the method of Atkinson and Morton (13).

RESULTS

Determination of uridine nucleotides. Changes induced in the concentrations of acid-soluble pyrimidine nucleotides of rat liver should be reflected in the absorption spectrum of the total acid-soluble fraction after the removal of purines. It was observed that the absorbancy of purine-free extracts from rats fed orotic acid was nearly twice as great in the region of 260 mμ as similar extracts from control rats. When the absorbancy of a purine-free extract from a control rat was subtracted from the absorbancy of a similar purine-free extract from an orotic acid-fed rat, a characteristic difference spectrum was obtained. Typical difference spectra obtained at pH 1 and pH 11 are shown in figure 1. The spectral constants for such typical difference spectra are given in table 1, as well as similar constants for

[3] Obtained from Pabst Laboratories, Milwaukee, Wisconsin.

TABLE 1

Comparison of spectral constants for liver difference spectra with reference compounds

	pH 1				pH 11				$\frac{A_{262}\ (pH\ 1)}{A_{262}\ (pH\ 11)}$
	λ_{max}	$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$	λ_{max}	$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$	
	mμ				mμ				
Difference spectra [1]	262	0.72	0.31	0.03	262	0.77	0.32	0.02	1.17
Reference compounds									
Uridine	262	0.74	0.33	0.03	262	0.83	0.29	0.02	1.19
Cytidine	280	0.45	2.10	1.58	271	0.86	0.94	0.34	
Thymidine	267	0.65	0.72	0.23	267	0.75	0.67	0.16	
Orotidine	267	0.65	0.79	—	267	0.82	0.72	—	
Orotic acid	280	0.55	1.80	1.55	286	0.79	1.73	1.73	
Uracil	259	0.84	0.17	0.01	284	0.71	1.40	1.27	
Cytosine	276	0.48	1.53	0.78	267	0.78	0.58	0.08	
Thymine	264	0.67	0.53	0.09	291	0.65	1.31	1.41	

[1] See figure 1. Constants for reference compounds were obtained from the literature (12).

known pyrimidine derivatives. Thus the increased light absorption of purine-free extracts of livers from rats fed orotic acid appears to result almost exclusively from uridine-containing components. It was therefore considered valid to use such difference spectra to quantify changes in the hepatic concentrations of uridine-containing nucleotides. Details of the method are presented in the Experimental Procedures section.

Changes in hepatic nucleotide levels. The changes induced in the levels of hepatic nucleotides by orotic acid feeding as well as the rapidity of reversal by addition of dietary adenine are shown in figure 2. The concentration changes were quite marked after only 16 hours of orotic acid feeding and a new equilibrium level was reached within 3 to 4 days, at which time lipid accumulation in the liver began (2,4). Further concentration changes observed after 4 days of orotic acid feeding resulted from dilution by the accumulating fat. The depression in total acid-soluble adenine was much more than could be accounted for by the depression of NAD + NADP and appears to represent a general depression of all adenine nucleotides. The concentration of adenosine-5′-triphosphate (ATP), measured enzymatically (14), was generally found to be depressed in approximately the same proportion as the total acid-soluble adenine. As previously reported by von Euler et al. (6), orotic acid feeding quickly resulted in a fourfold increase in the concentration of acid-soluble uridine but, as indicated by the spectral evidence above, there was no comparable accumulation of orotic acid or other pyrimidines. Also the altered nucleotide concentrations were very rapidly returned to normal by the addition of small amounts of adenine sulfate to the diet (fig. 2).

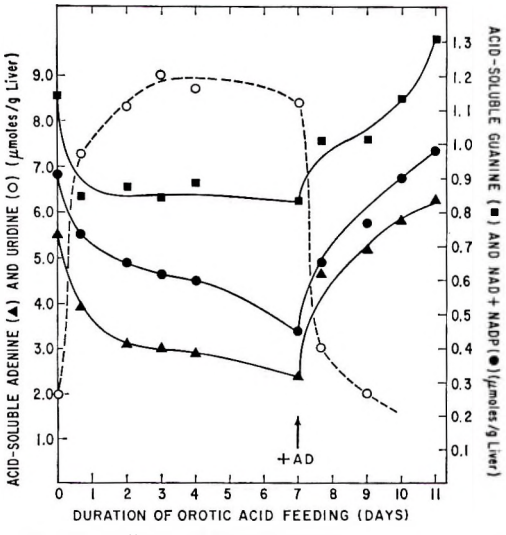

Fig. 2 Effect of dietary orotic acid and adenine sulfate supplementation on the concentrations of acid-soluble nucleotides of rat liver. Young rats (40 to 50 g) were fed basal diet R1FF for 10 days. Their diet was then supplemented with 1% orotic acid and they were killed on the day indicated. After 7 days, the diet was further supplemented with 0.25% adenine sulfate. The data are from a total of 85 animals, with at least 4 per point. The extraction and analysis of nucleotides are described under Experimental Procedures.

Effect of dietary supplementation with pyridine derivatives. The ability of large dietary supplements of niacin to elevate hepatic pyridine nucleotide levels is well known (15). The effect of niacin and nicotinamide feeding on the fatty liver and altered nucleotide levels induced by orotic acid was therefore examined (table 2). Nicotinic acid and nicotinamide alone (groups C, E, and G) did not alter the hepatic fat content but they did produce a twofold increase in liver NAD + NADP concentrations and a corresponding increase in the concentration of acid-soluble adenine. When added to a diet containing 1% orotic acid, neither nicotinic acid (1%) (group D) nor nicotinamide (0.2%) (group F) was very effective in preventing the fatty liver; however, orotic acid largely prevented the increase in pyridine nucleotides which these compounds alone induce. The rats in groups D and F developed fatty livers which had a normal, or nearly normal, amount of NAD + NADP but in which the total acid-soluble adenine and guanine concentrations were still depressed. This suggests that hepatic fat accumulation is more closely related to a low purine nucleotide level than to low concentrations of pyridine nucleotides.

Increasing the nicotinamide supplement to 1% (group H) prevented the orotic acid-induced fatty liver. However, this amount of nicotinamide reduced the food consumption, which alone could account

TABLE 2

Effect of dietary orotic acid (OA), nicotinamide (NAM) and nicotinic acid (NA) on liver lipid and nucleotide levels

Group [1]	Additions to basal diet (W-4)	Diet consumed	Liver analyses [2]			
			Total lipid	NAD + NADP	Acid-soluble adenine	Acid-soluble guanine
		g/day	*%*	*μmoles/g*	*μmoles/g*	*μmoles/g*
A	None	14.2	6.2 ± 0.9	0.78 ± 0.05	3.89 ± 0.06	0.48 ± 0.02
B	1% OA	14.2	29.1 ± 1.0	0.30 ± 0.02	1.64 ± 0.42	0.31 ± 0.06
C	1% NA	13.0	6.9 ± 0.6	1.49 ± 0.09	4.58 ± 0.15	0.50 ± 0.03
D	1% OA + 1% NA	14.3	16.1 ± 3.2	0.75 ± 0.06	2.07 ± 0.17	0.35 ± 0.01
E	0.2% NAM	13.4	7.9 ± 0.6	1.46 ± 0.20	4.36 ± 0.34	0.52 ± 0.04
F	1% OA + 0.2% NAM	11.1	23.7 ± 4.6	0.51 ± 0.07	1.63 ± 0.24	0.36 ± 0.03
G	1% NAM	6.0	5.8 ± 0.2	2.78 ± 0.84		
H	1% OA + 1% NAM	5.3	7.1 ± 1.1	1.22 ± 0.12		

[1] The respective diets were fed for 14 days before the livers were analyzed. Rats were of the Osborne-Mendel strain (in this experiment only).
[2] Each figure is the mean of 4 to 8 animals ± SE.

for the failure to observe any liver fat accumulation.

Pyridine nucleotide synthesis from injected precursors. The results in table 2 suggested that orotic acid was inhibiting pyridine nucleotide synthesis in rat liver. This possibility was examined further by injecting various pyridine nucleotide precursors and measuring accumulation of hepatic NAD + NADP. In figure 3, curve A shows the response of rat liver pyridine nucleotides to a single intraperitoneal injection of nicotinamide. A threefold elevation was observed after 8 hours, followed by a rapid return to normal. Rats fed 1% orotic acid for 3 days (curve B) had less liver NAD + NADP and responded only slightly to a single injection of nicotinamide. The peak in pyridine nucleotide accumulation was observed after 3 hours. Rats fed 1% orotic acid plus 0.25% adenine sulfate (curve C) had nearly normal hepatic concentrations of NAD + NADP, and for 3 hours after a nicotinamide injection their livers accumulated additional coenzymes at the normal rate. After 3 hours, however, the concentration decreased. The NAD + NADP accumulation was markedly stimulated in the rats fed orotic acid when nicotinamide injection was accompanied by administration of adenosine (curve D). The effectiveness of other adenine derivatives when administered as a single dose with nicotinamide was also tested (table 3). Both ATP and adenine sulfate, as well as adenosine, were partially effective in stimulating

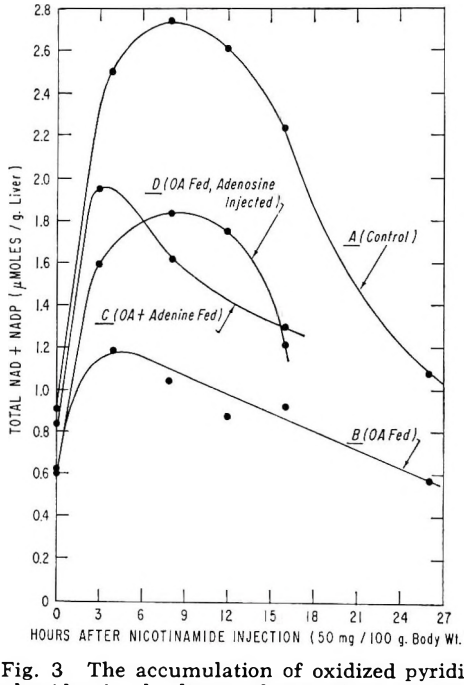

Fig. 3 The accumulation of oxidized pyridine nucleotides in the livers of rats given an intraperitoneal dose of nicotinamide. Young rats (50-60 g) were fed a basal diet (W-4) for 7 to 10 days. Then for 72 hours, preceding the nicotinamide administration, the diet was supplemented, as follows: *A*, none; *B*, 1% orotic acid; *C*, 1% orotic acid plus 0.25% adenine sulfate; *D*, 1% orotic acid. In addition to nicotinamide, rats in group *D* were injected with 40 mg of adenosine/100 g body weight in 4 equal portions at 0, 2.5, 5.0 and 7.5 hours after the nicotinamide injection. The data are from a total of 145 animals, with 4 to 21 per point. The pyridine nucleotides were analyzed as described under Experimental Procedures.

TABLE 3

Hepatic pyridine nucleotide accumulation following the administration of nicotinamide and various adenine derivatives

Additions to basal diet (W-4)	Compounds injected [1]	Liver NAD + NADP 8 hours after injections
		μmoles/g liver
None	none	0.91 ± 0.03 (19) [2]
	ATP	0.98 ± 0.02 (3)
	NAM	2.74 ± 0.18 (14)
	NAM + ATP	2.49 ± 0.27 (4)
1% Orotic acid [3]	none	0.63 ± 0.02 (21)
	ATP	0.89 ± 0.07 (3)
	NAM	1.05 ± 0.06 (10)
	NAM + ATP	1.95 ± 0.23 (4)
	NAM + adenine sulfate	1.51 ± 0.13 (4)
	NAM + adenosine	1.84 ± 0.34 (4)

[1] Injections were intraperitoneal in 1 ml or less of 0.95% NaCl. The amount given, in mg/100 g body weight, was as follows: nicotinamide (NAM), 50; disodium ATP·$4H_2O$ (ATP), 100; adenine sulfate, 30; adenosine, 40 (in four 10-mg doses at 0, 2.5, 5.0 and 7.5 hours after the NAM injection). Livers were analyzed 8 hours after the NAM injections.

[2] Mean ± SE. Numbers in parentheses indicate number of animals.

[3] One per cent of orotic acid was added to the diet 72 hours before the rats were injected.

hepatic pyridine nucleotide accumulation in orotic acid-fed rats. The ATP had no effect on the nicotinamide response of normal rats, confirming the observation of Minard and Hahn (16).

In figure 4 a comparison is made of accumulation of pyridine nucleotides and of total acid-soluble adenine in livers of rats from groups A and C in figure 3. Adenine and pyridine nucleotides accumulate in equimolar amounts in normal nicotinamide-injected rats. In orotic acid-fed rats given nicotinamide and adenosine, accumulation of hepatic adenine was found to be more than sufficient to account for the increase of pyridine nucleotides. These data indicate that adenosine administered parenterally to orotic acid-fed rats can expand the pool of acid-soluble adenine. In comparable animals, nicotinamide alone resulted in little elevation of either pyridine nucleotides or of acid-soluble adenine (see table 2).

The effectiveness of nicotinic acid mononucleotide (NaMN) as a precursor of hepatic pyridine nucleotides in control and in orotic acid-fed rats is shown in figure 5. As reported by Minard and Hahn (16), control rats converted NaMN rapidly and efficiently to hepatic pyridine nucleotides. In rats fed orotic acid for 3 days,

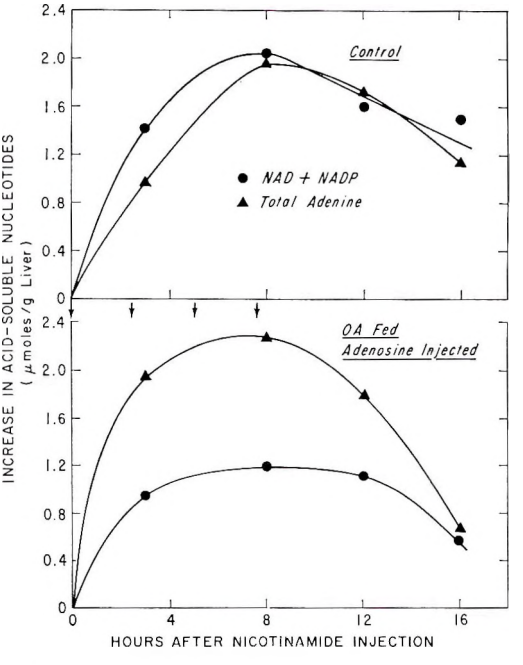

Fig. 4 The increase in hepatic NAD + NADP and total acid-soluble adenine following the intraperitoneal administration of nicotinamide. The data are from rats in groups *A* and *D* of figure 3. The methods of analysis are described under Experimental Procedures. The arrows indicate the times when adenosine was injected.

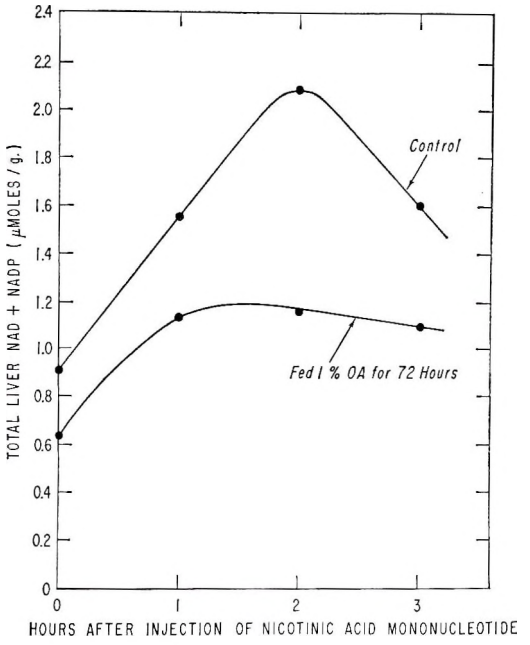

Fig. 5 The accumulation of oxidized pyridine nucleotides in the liver of rats given an intraperitoneal dose of nicotinic acid mononucleotide. Young rats (50 to 60 g) were fed a basal diet (W-4) for 7 days. Then the diet of some of the rats was supplemented with 1% orotic acid for 72 hours, after which all rats received an intraperitoneal injection of NaMN (29.3 μmoles/100 g body weight). At hourly intervals following injection, groups of rats were killed and their livers analyzed for NAD + NADP, as described under Experimental Procedures. Each point is the average of 3 to 4 rats.

only a very modest accumulation was observed which reached a maximum after only one hour. In another experiment, 4 rats fed 1% orotic acid and 0.25% adenine sulfate for 3 days showed only a slightly greater response to a NaMN injection than did rats fed only orotic acid. Their hepatic acid-soluble adenine concentration before injection was 4.3 μmoles/g as compared with 2.9 μmoles/g for orotic acid-fed rats. The control rats in figure 5 had 5.2 μmoles/g of hepatic acid-soluble adenine before injection with NaMN.

DISCUSSION

The method described for determining the acid-soluble adenine and guanine concentrations has been applied to yeast (17) and appears to be applicable to liver. Using the equations of Loring et al. (9), the purines can be determined using absorbancy values at 262 mμ and at 280 mμ or at 262 mμ and 240 mμ. In liver, adenine determinations using the 2 sets of values were nearly identical, although there was usually more discrepancy in the case of guanine (± 12.8%). Guanine was therefore determined by averaging the 2 results as well as a third estimate obtained by subtracting the adenine from the total purine concentration obtained from the absorbancy at 252 mμ and at 276 mμ (9). The method is useful for rapidly assaying a large number of samples for total adenine and guanine; and, because quantitative separation of purines is achieved, it may permit spectrophotometric analysis of changes in non-purine components, as in the present study.

As early as 16 hours after the ingestion of orotic acid, the concentration of acid-soluble uridine derivatives in the liver is elevated and a progressive decrease of purine and pyridine nucleotide concentrations is noted which reaches a minimum on the third or fourth day. This decrease coincides with a decrease of even greater proportion in the concentrations of plasma triglycerides, phospholipids, and cholesterol, a result of impaired hepatic lipoprotein secretion (3,4). Also on the third or fourth day the hepatic accumulation of triglycerides and cholesterol begins, and it proceeds for 10 to 14 days to a final liver fat content which is more than 10 times normal (2,4).

Once the new equilibrium levels of hepatic nucleotides and plasma and liver lipids have been established, they remain virtually unchanged over many weeks and even months of orotic acid feeding. Thus low hepatic concentrations of adenine and guanine nucleotides (7) as well as pyridine nucleotides (18) have been observed after 4 weeks, and depressed plasma lipids[4] and elevated uridine nucleotides and liver fat (6) after nearly a year of orotic acid ingestion. Despite these large deviations from the normal, under standard laboratory conditions the rats grow to maturity and appear healthy.

Adenine, when added in small amounts to diets containing orotic acid, rapidly re

[4] Windmueller, H. G., unpublished observations.

stores concentrations of liver nucleotides (fig. 2) and plasma lipids (3,4) to normal. The exogenous adenine may function by supplementing endogenous purines, whose synthesis has been impeded or utilization accelerated. This explanation, however, does not readily account for the ability of exogenous adenine to correct the orotic acid-induced elevation of uridine nucleotides. Therefore, in addition to replacing endogenous purines, exogenous adenine may be inhibiting the otherwise rapid conversion of orotic acid to uridylic acid (19). In the supernatant fraction of rat liver, small amounts of adenine can inhibit this conversion when the availability of 5-phosphoribosyl-1-pyrophosphate (PRPP) is limiting (6). In the whole animal as well, supplementary adenine partially inhibits this conversion (20).

Rajalakshmi et al. (21), in a communication which appeared during the course of this work, reported an inhibition of hepatic pyridine nucleotide synthesis from nicotinamide in rats with very fatty livers produced by 4 weeks of orotic acid feeding. Depressed concentrations of pyridine nucleotides have likewise been reported in fatty livers produced by other dietary means (22, 23). From the data in figure 3, however, it is evident that nicotinamide ceases to stimulate hepatic pyridine nucleotide accumulation in rats fed orotic acid for only 72 hours, before any liver fat accumulation is evident. Two hypotheses were considered to explain this inhibition.

1) In the rat, NAD synthesis from nicotinamide begins with deamidation to nicotinic acid (24), followed by the reaction of nicotinic acid with PRPP to give NaMN, a reaction requiring catalytic amounts of ATP (25). NaMN synthesis could become inhibited when large amounts of PRPP are diverted for the conversion of orotic to orotidylic acid. However, the observation that NaMN itself is a poor precursor for pyridine nucleotides in orotic acid-fed rats (fig. 5) fails to support this hypothesis.

2) NAD synthesis may be depressed by low concentrations of adenine nucleotides, particularly ATP. Considerable support may be found for this hypothesis. (a) Adenosine administration expands the hepatic pool of acid-soluble adenine and also stimulates NAD synthesis (fig. 3, table 3). (b) Nakamura et al. (26) have recently described an enzyme from rat liver which acts as a NaMN phosphatase when ATP concentrations are low and as a NaMN pyrophosphorylase, synthesizing NaMN from nicotinic acid and PRPP, when ATP concentrations are high. Thus the concentration of ATP could regulate NAD formation. High NaMN phosphatase activity, resulting from a low ATP concentration, would also explain the poor precursor activity of NaMN in our experiments (fig. 5). (c) Depressed NAD synthesis from nicotinamide can be correlated with lowered hepatic ATP concentrations in ethionine-treated rats (27). It may be pertinent that ethionine-treated rats also develop a fatty liver. (d) In mice, 6-mercaptopurine, an adenine antagonist, inhibits NAD synthesis from nicotinamide and the inhibition is relieved by yeast adenylic acid (28). And, finally, (e) in cultures of *Escherichia coli* and *Bacillus subtilis*, adenine nucleotides appear to have a regulatory function in the control of NAD synthesis (29).

The reason for the depressed hepatic adenine nucleotide concentration in orotic acid-fed rats and its relation to hepatic lipoprotein secretion remain to be established.

ACKNOWLEDGMENT

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Mineral Utilization in the Rat

IV. EFFECTS OF CALCIUM AND PHYTIC ACID ON THE UTILIZATION OF DIETARY ZINC

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ABSTRACT Weanling rats were used in a series of growth and mineral balance experiments to study effects of dietary calcium and phytic acid on the utilization of zinc, magnesium, calcium and phosphorus in semi-purified diets. Dietary calcium decreased zinc absorption from diets containing phytic acid but did not significantly lower the amount of zinc absorbed from diets without phytic acid. Magnesium and phosphorus absorption were decreased by extra calcium, in the presence or absence of phytic acid. Dietary phytic acid lowered magnesium absorption and, in some instances, decreased the absorption of zinc. Phytic acid-phosphorus was readily utilized by the weanling rat.

In an earlier study carried out in this laboratory (1) it was observed that an increased level of dietary calcium decreased the percentage zinc absorption in the presence of soy protein but did not affect zinc absorption when whole egg white served as the protein source. Thus, it appeared that the effect of calcium on zinc utilization is to depress absorption and that a third dietary variable, probably involving phytic acid mediates this interaction. The experiments reported in the present paper were designed to study this observation further. In addition, the effects of dietary calcium and phytic acid on the absorption of magnesium, calcium and phosphorus were investigated.

METHODS

In the 4 experiments reported in this paper, 20-day-old (approximately 50 g) male albino rats of the Sprague-Dawley strain were alloted at random, in an ungrouped randomized design, to individual cages in a stainless steel battery for 18 days. Room temperature was maintained near 22°. Feed and demineralized water were supplied ad libitum. The feed consumption and weight gains were recorded. Feces, and urine in experiment 4, were collected for a 6-day period beginning the seventh day on trial. The rats were decapitated after the eighteenth day and, in experiments 2, 3, and 4, the femur bones were removed for zinc analysis. Diets, feces, femurs and urine were analyzed for zinc by the method of Butts et al. (2). Diets and feces collected in experiments 2, 3 and 4 were further analyzed for total phosphorus by a method adapted from Rice (3), and for calcium and magnesium by the chelometric method of Malmstadt and Hadjiioannou (4). Prior to the calcium and magnesium determinations, phosphorus and interfering cations were removed by use of cyclohexanediaminetetraacetic acid on acetate-saturated Dowex 21K (5). Phytic acid content of the diets was determined by a method adapted from Holt (6).

The basal diet (table 1) contained approximately 4 ppm Zn. Additional zinc was added to all diets as $ZnCO_3$. The 0.4% phytic acid diets fed in experiment 1 were prepared by adding a solution of phytic acid [2] directly to the zinc-supplemented basal diet. In the preparation of 2.0% phytic acid diets (exps. 2, 3 and 4) $CaHPO_4$ and $NaH_2PO_4 \cdot H_2O$ were omitted from the basal diet, since the addition of phytic acid supplied adequate phosphorus to meet the animals' requirement for this element. Calcium was added as $CaCO_3$.

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[2] Phytic acid, Nutritional Biochemicals Corporation, Cleveland. Contained by analysis 25 to 50% phytic acid. Each lot varied and was analyzed before adding to the diet in amount required to give desired concentration.

TABLE 1

Composition of basal diet

	%
Glucose monohydrate [1]	62.5
Casein [2]	15.0
DL-Methionine	0.3
Corn oil	10.0
Cellulose [3]	3.0
Mineral mixture [4]	1.1
$CaHPO_4$	1.4
$NaH_2PO_4 \cdot H_2O$	1.2
Vitamin-glucose mixture [5]	5.0
Vitamin A and D concentrate [6]	0.5

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Vitamin-Free Test Casein, General Biochemicals Incorporated, Chagrin Falls, Ohio.
[3] Solka-Floc, Brown Company, Chicago.
[4] Forbes and Yohe (11) less the $CaHPO_4$.
[5] Forbes and Yohe (11) less the chlortetracycline.
[6] Standardized Cod Liver Oil, Parke Davis and Company, Detroit, 2000 IU vitamin A and 250 IU vitamin D/g.

In experiments 1 and 2 all diets contained 12 ppm Zn as did the 0.8% Ca diet in experiment 3; all other diets in experiments 3 and 4 contained 65 ppm Zn. All diets contained 0.04% Mg; the phytate-free diets contained 0.74% P, and those with phytic acid added contained 0.94% P.

Four animals were used in each treatment. Analysis of variance accompanied by the *t* test, as given in Goulden (7), was used to test the data statistically. Differences referred to in the text possess a probability value of less than 0.05.

RESULTS AND DISCUSSION

The data in table 2 show that addition of 2.0% phytic acid interfered with weight gain when the Zn supply was at the requirement level (exp. 2) and not when excess Zn was present (exps. 3 and 4). Furthermore, this effect of phytic acid was greater at the 2 higher levels of dietary calcium. Addition of 0.4% phytic acid did not affect weight gain in spite of a marginal supply of zinc in experiment 1. Over all of the experiment, weight gain was a reflection of amount of feed consumed, the correlation coefficient being 0.965.

During the collection periods the average feed intake was 10 to 11.5 g daily except for the animals receiving phytic acid in experiment 2. These rats consumed an average of 7.7, 5.0 and 4.7 g of feed daily at 0.4, 0.8 and 1.2% Ca, respectively, and exhibited typical signs of Zn deficiency.

It is well established that zinc is less readily available from plant protein, e.g., soy and sesame, than from animal protein diets. Further work with chicks (8) pigs [3] and rats (9) has shown that the addition of phytic acid to diets decreases the availability of dietary zinc. The addition of either 0.4 or 2.0% phytic acid to diets used in the present experiments similarly decreased the amount of zinc absorbed when the diets contained 12 ppm Zn, but did not lower zinc absorption from diets containing 69 ppm Zn, as shown in table 3. Rats fed 12 ppm Zn diets with 2.0% phytic acid (exp. 2) were markedly zinc-deficient, as indicated by their growth rate. Zinc deficiency symptoms were not evident, however, when rats were fed 12 ppm Zn diets containing 0.4% phytic acid (exp. 1). Since 0.4% phytic acid is a level of phytic acid one might expect to find in a plant protein diet, it appears that factors other than, and possibly independent of, phytic acid affect the availability of zinc

[3] Oberlease, D., M. E. Muhrer and B. L. O'Dell 1960 Some effects of phytic acid on zinc availability and physiology in swine. J. Animal Sci., *19:* 1280 (abstract).

TABLE 2

Daily gain in weight of rats from 20 to 38 days of age

Diets [1]	Exp. 1 [2,3]	Exp. 2	Exp. 3	Exp. 4
	g	*g*	*g*	*g*
0.4% Ca	4.8 ± 0.35 [4]	4.8 ± 0.10		5.2* ± 0.47
0.4% Ca + phytic acid	3.7 ± 1.05	2.6 ± 0.36	3.8* ± 0.22	4.2* ± 0.32
0.8% Ca	4.5 ± 0.17	4.6 ± 0.13	4.4 ± 0.20	5.0* ± 0.25
0.8% Ca + phytic acid	4.4 ± 0.25	1.1 ± 0.18	3.9* ± 0.20	4.4* ± 0.32
1.2% Ca	4.4 ± 0.25	4.3 ± 0.35		5.2* ± 0.20
1.2% Ca + phytic acid	4.2 ± 0.05	0.9 ± 0.14	3.9* ± 0.30	5.1* ± 0.51

[1] Four rats/treatment.
[2] Phytic acid diets in experiment 1 contained 0.4% phytic acid. In all other experiments they contained 2.0% phytic acid.
[3] All rats received 12 ppm dietary Zn except those marked (*) which received 65 ppm Zn.
[4] Mean ± SD.

TABLE 3

Mineral absorption by rats fed diets containing calcium or calcium plus phytic acid

Diet [1]	Exp. 1	Exp. 2	Exp. 3	Exp. 4
	%	%	%	%
		Zinc		
0.4% Ca	70 ± 1.8 [2] (8.8) [3]	70 ± 2.7(8.4)		29 ± 6.0(18.7)
0.4% Ca + phytic acid	63 ± 6.1 (8.0)	50 ± 9.5(5.9)	28 ± 8.9(19.1)	31 ± 7.2(20.5)
0.8% Ca	64 ± 2.4 (7.8)	72 ± 3.0(8.6)	69 ± 3.4(8.9)	28 ± 3.0(18.5)
0.8% Ca + phytic acid	62 ± 6.2 (7.7)	18 ± 5.7(2.1)	21 ± 3.5(14.5)	22 ± 6.1(14.3)
1.2% Ca	64 ± 7.3 (7.8)	66 ± 5.3(7.8)		22 ± 2.5(14.5)
1.2% Ca + phytic acid	54 ± 3.9 (6.7)	13 ± 9.0(1.5)	18 ± 3.7(12.6)	17 ± 4.8(11.4)
		Magnesium		
0.4% Ca		81 ± 5.2		76 ± 4.7
0.4% Ca + phytic acid		69 ± 5.9	65 ± 6.2	66 ± 5.2
0.8% Ca		69 ± 4.0	66 ± 7.6	66 ± 5.3
0.8% Ca + phytic acid		46 ± 4.3	50 ± 4.3	57 ± 5.8
1.2% Ca		58 ± 7.5		56 ± 6.6
1.2% Ca + phytic acid		43 ± 11.8	43 ± 7.7	49 ± 3.4
		Calcium		
0.4% Ca		82 ± 2.7(0.32)		77 ± 1.5(0.32)
0.4% Ca + phytic acid		78 ± 6.5(0.31)	80 ± 4.6(0.32)	79 ± 4.9(0.33)
0.8% Ca		60 ± 4.3(0.50)	59 ± 4.2(0.43)	56 ± 5.7(0.45)
0.8% Ca + phytic acid		45 ± 9.3(0.37)	50 ± 2.1(0.42)	58 ± 3.9(0.48)
1.2% Ca		45 ± 6.4(0.51)		42 ± 5.3(0.49)
1.2% Ca + phytic acid		39 ± 11.0(0.44)	40 ± 2.9(0.46)	48 ± 2.2(0.56)
		Phosphorus		
0.4% Ca		93 ± 1.6(0.69)		90 ± 1.7(0.68)
0.4% Ca + phytic acid		87 ± 3.6(0.84)	88 ± 1.7(0.84)	87 ± 2.2(0.83)
0.8% Ca		76 ± 3.6(0.56)	74 ± 5.2(0.55)	80 ± 1.5(0.61)
0.8% Ca + phytic acid		62 ± 5.6(0.60)	66 ± 2.2(0.63)	72 ± 0.6(0.68)
1.2% Ca		64 ± 3.3(0.47)		63 ± 3.6(0.48)
1.2% Ca + phytic acid		54 ± 7.2(0.52)	56 ± 1.7(0.54)	60 ± 2.4(0.58)

[1] Four rats/treatment.
[2] Mean ± SD.
[3] Numbers in parentheses indicate parts per million of absorbable Zn or percentage of absorbable Ca or P in the diet.

in plant protein diets. The manner in which phytic acid is added to the diet might also be of importance (8).

The addition of extra calcium to diets without phytic acid (control diets) did not, at any time, significantly decrease the percentage of zinc absorption. Increasing the calcium level from 0.4 to 1.2% did, however, decrease the amount of zinc absorbed from diets containing phytic acid. In some instances with phytic acid diets (exps. 2 and 4) the percentage of zinc absorption was significantly lowered by increasing the calcium level from 0.4 to 0.8%. Decreased zinc absorption resulting from increased calcium levels when soybean protein diets were fed to rats has been noted by Heth et al. (10) and by Forbes (1). Diets used by Heth et al. contained 0.17, 0.77 and 1.93% calcium, whereas Forbes fed diets containing 0.4 and 0.8% calcium. In an earlier study with rats fed casein and soybean diets containing 0.8% and 1.6% calcium, Forbes and Yohe (11) reported that zinc absorption did not decrease at the higher level of calcium. It appears that rat diets containing soybean or containing casein + phytic acid, must have an initial level of dietary calcium below 0.8% before extra calcium will result in a significant decrease in the percentage of zinc absorption. This initial level of dietary calcium may not, however, be as critical in other species such as the chick (12).

Approximately 3.1% of the dietary zinc was excreted by way of the urine in experiment 4. The percentage of urinary zinc was not affected by either phytic acid or the level of dietary calcium. In a study with chicks, Savage et al. (13) similarily noted that phytic acid has little or no effect

TABLE 4

Ash and zinc in the femur of rats fed diets containing calcium or calcium plus phytic acid at various levels

Diet [1]	Exp. 2	Exp. 3	Exp. 4
	Ash in dry lipid free femur, %		
0.4% Ca	50 ± 0.6 [2]		57 ± 3.9
0.4% Ca + phytic acid	50 ± 0.6	55 ± 0.6	56 ± 3.1
0.8% Ca	53 ± 1.0	57 ± 1.0	56 ± 1.4
0.8% Ca + phytic acid	51 ± 1.1	56 ± 1.8	56 ± 1.7
1.2% Ca	55 ± 0.6		59 ± 1.5
1.2% Ca + phytic acid	50 ± 0.6	56 ± 1.0	57 ± 0.8
	Zinc in femur ash, ppm		
0.4% Ca	401 ± 26		458 ± 40
0.4% Ca + phytic acid	307 ± 52	396 ± 38	405 ± 38
0.8% Ca	360 ± 49	335 ± 24	365 ± 23
0.8% Ca + phytic acid	233 ± 22	368 ± 31	296 ± 13
1.2% Ca	298 ± 12		323 ± 46
1.2% Ca + phytic acid	259 ± 24	318 ± 16	261 ± 13

[1] Four rats/treatment.
[2] Mean ± SD.

on the percentage of urinary zinc excretion.

In experiment 2, table 4, small but significant ($P < 0.01$) differences in the percentage of femur ash were observed between zinc-deficient rats and their controls as a result of increase in femur ash as dietary calcium increased in non-phytate diets but not in phytate-containing diets. However, no difference in the percentage of femur ash between zinc-deficient chicks and their controls was detected in an earlier study carried out in this laboratory (14). It appears that zinc deficiency may result in lower levels of ash in the dry, lipid-free femur, but the difference, if present, is small.

Rats fed phytic acid diets in experiments 2 and 4 had a lower concentration of zinc in their femur ash than rats fed the control diets. Concentration of zinc in femur ash was also decreased by increasing the calcium level of control and phytic acid diets from 0.4 to 1.2%. The correlation between absorbed Zn (ppm) and parts per million of Zn in femur ash was determined from the data of experiments 2 and 4. The coefficients are, respectively, 0.76 and 0.62, and are highly significant ($P < 0.01$). However, the correlation coefficient obtained using all 64 rats in experiments 2, 3 and 4 is only 0.06, which is not significant. This latter low correlation between absorbed Zn (ppm) and parts per million of Zn in femur ash resulted since rats fed diets containing 67 ppm Zn absorbed more than twice as much zinc as rats fed diets containing 12 ppm Zn, and the increase in zinc absorption was accompanied by only a slight increase in concentration of zinc in femur ash. Concentration of zinc in femur ash appears to be governed by the level of dietary calcium, which may or may not affect zinc absorption, as well as by the zinc content of a diet.

Rats fed 2.0% phytic acid diets absorbed less magnesium than rats fed control diets (table 3). Similar results have been obtained by McWard and Imondi [4] who, using chick growth and mortality as criteria, observed that the availability of magnesium from isolated soybean protein diets was decreased by the addition of phytic acid to the diets. The fact that absorption of magnesium from both the control and phytic acid diets was decreased when the level of dietary calcium increased from 0.4 to 1.2% is in agreement with earlier work by McAleese and Forbes (15) and Forbes (16).

Results of this study were not definite enough to permit conclusions as to the effect of 2.0% phytic acid on the availability of calcium (table 3). In experiment 2 zinc-deficient rats fed phytic acid diets absorbed less dietary calcium than

[4] McWard, G. W., and A. R. Imondi 1963 The effects of phytic acid and EDTA on the chick's requirement for magnesium. Poultry Sci., 42: 1290 (abstract).

rats fed control diets, whereas in experiment 4, in which zinc deficiency symptoms were not apparent, the rats fed phytic acid diets tended to absorb more dietary calcium than their controls. It was noted, however, that an increase in absorbable calcium occurred when the level of calcium in control and phytic acid diets was increased from 0.4 to 1.2%.

Phosphorus in phytic acid diets was readily absorbed by the rat. As a result, rats fed phytic acid diets, which contained slightly more phosphorus than control diets, absorbed more phosphorus from each gram of diet than did rats fed control diets. Krieger et al. (14), using change in body weight and bone ash as criteria, reported that rats effectively utilized phytic acid phosphorus when diets contained adequate vitamin D, but in no case was utilization of phosphorus in phytic acid equal to that of inorganic phosphorus. A lower utilization of phytic acid phosphorus when compared with inorganic phosphorus may account for the fact that the percentage of absorption of phosphorus from phytic acid diets was slightly lower than the percentage absorption of phosphorus from the controls. However, at least part of this difference might also be accounted for by the higher levels of total phosphorus contained in the phytic acid diets. Extra calcium added to either control or phytic acid diets decreased phosphorus absorption as observed by Forbes (17). Assuming that nonphytic acid phosphorus was absorbed from phytic acid diets to the same extent as phosphorus in control diets, the average percentage of phosphorus absorbed from phytic acid in diets containing 0.4, 0.8 and 1.2% Ca was, respectively, 85, 59 and 51%.

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Urinary and Fecal Excretion of Endogenous Nitrogen by Infants and Children [1]

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ABSTRACT Urinary and fecal excretions of endogenous nitrogen were determined in metabolic balance studies with normal 4- to 6-month-old North American infants and well nourished but dwarfed 3- to 8-year-old African children. Data from these studies and from several studies of North American adults are analyzed. The importance of controlling prior intake of protein is stressed. When prior intake of protein had been "low or intermediate," mean urinary excretion of nitrogen by infants, children and adults was 37, 48 and 30 mg/kg/day, respectively, during the fourth through sixth days of ingesting the protein-deficient diet. Corresponding fecal excretions of nitrogen were 20, 25 and 11 mg/kg/day. When urinary excretion of endogenous nitrogen is expressed in relation to basal caloric consumption, the mean value for 4- to 6-month-old infants is only about one-half the mean value for adults (0.6 versus 1.2 mg/basal kcal). The data therefore do not offer support for the concept that urinary excretion of endogenous nitrogen is related in a fundamental physiologic manner to basal metabolic rate.

The requirement for protein includes 3 components (1, 2): (a) the amount necessary for replacement of urinary and fecal losses of endogenous nitrogen, (b) the amount necessary for integumental renewal, and (c) the amount necessary for growth. Urinary and fecal excretion of endogenous nitrogen may be considered to reflect to some extent the rate of nitrogen turnover in the body, whereas integumental renewal refers to replacement of nitrogen lost in sweat, in desquamated epithelium, in hair (of head, face and body), in fingernails and toenails. Since integumental renewal includes the requirement for "adult growth" (i.e., growth of hair and nails), the third component mentioned above applies primarily to children.

The basis for a theoretic estimate of protein requirement appears sound. If precise figures could be provided for each of the 3 components, the true requirement could be stated with considerable confidence. Without precise data, calculations based on the best available data and certain assumptions may provide an approximation of the truth.

Excretion of endogenous nitrogen is generally considered (3, 4) to be the lowest excretion attained after an empirically defined interval during which the organism ingests a diet low in nitrogen but complete and abundant with respect to other nutrients. A considerable body of data has been accumulated concerning excretion of endogenous nitrogen by laboratory animals, domestic animals and adult human subjects, but little information is available concerning human infants and children.

Urinary excretion of endogenous nitrogen by animals of various species, from those of small size and relatively high metabolic rate (e.g., mouse) to those of large size and relatively low metabolic rate (e.g., cow, elephant), appears to be remarkably constant when expressed in relation to basal energy expenditure. With few exceptions, the value is approximately 2 mg of endogenous nitrogen/basal kcal (3, 4). On the basis of meager data it has been concluded (3, 4) that the value for human adults is also approximately 2 mg/basal kcal and this figure has frequently

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been cited in the literature. However, as will be discussed, the most satisfactory studies of human adults (5–7) suggest that a value of 1.2 to 1.5 mg/basal kcal is more appropriate.

If urinary excretion of endogenous nitrogen were related to energy metabolism in some fundamental manner, it might be anticipated that similar rates of excretion per basal calorie would apply for human infant, child and adult. Since values for urinary excretion of nitrogen per basal calorie are shown in the present study to be less for infants and children than those reported from studies of human adults, the data cast doubt on the physiologic significance of the relation between urinary excretion of endogenous nitrogen and basal energy expenditure.

SUBJECTS AND FEEDINGS

Four- to six-month-old infants. One 3-day nitrogen balance study was performed with each of 19 normal full-term infants (12 males and 7 females) ranging in age from 125 to 187 days at the time of study. Birth weight, sex, age and data from metabolic balance studies are presented in table 1. All infants had lived at home but 12 (those listed in table 1(a) and infants S.We., T.Si., K.Wo. and K.Ir.) had each been admitted to the metabolism ward to serve as subjects for a 3-day metabolic balance study during each 2-week interval from birth until the time of the studies reported here. In a sense, these infants could be considered trained subjects.

Previous dietary history: From birth until institution of the protein-deficient diet, 15 infants (table 1 (a and b)) had been fed ad libitum with formulas providing 1.5 to 1.7 g of protein/100 ml. A milk formula had served as sole source of energy until 2 to 3 weeks before instituting the protein-deficient diet. Intakes of protein by these infants during the 3 weeks before institution of the protein-deficient diet had averaged approximately 2.5 g/kg/day. At the time of institution of the protein-deficient diet, they had been receiving small amounts of precooked cereal and commercially prepared strained foods for infants. The remaining infants (table 1 (c)) had received evaporated milk and water without additional carbohydrate (protein concentration: 3.4 g/100 ml) from birth until institution of the protein-deficient diet. Cereal and a variety of strained foods had been introduced into the diet beginning at about 2 months of age. Intakes of protein by these infants during the 3 weeks before institution of the protein-deficient diet had averaged approximately 4.5 g/kg/day.

Protein-deficient diets: Each infant received a protein-deficient diet from 3 days before institution of the metabolic balance study described in this report until completion of the 3-day study. The diet consisted of a formula low in protein and, in some instances, of strained pears and bananas, all fed ad libitum. The formula provided 67 kcal/100 ml and contained small amounts of protein and non-protein nitrogen from cow's milk together with lactose, corn oil or butterfat, calcium and phosphorus. It seemed unlikely that the 6-day interval of protein deprivation imposed only once during the first 6 months of life would be detrimental to the infants.

Three- to eight-year-old African children. Fifteen children of the Bashi tribe, 8 boys and 7 girls, were studied by one of the authors (EMD) at the Institut pour la Recherche Scientifique en Afrique Centrale (IRSAC), Lwiro/Bukavu, Republic of the Congo. These children ranged in age from 3 years 3 months to 7 years 10 months. With the exception of one normal child (no. 147), all had recovered from minor parasitic infections, infectious diseases, mild malnutrition or kwashiorkor. Detailed information concerning 14 of these subjects has been presented previously (8). The fifteenth child (no. 208) had been admitted to the Research Center at age 4 years 6 months for treatment of mild malnutrition. Weight at the time of admission was 11.85 kg and height was 87.0 cm. All of the children had received adequate diets for at least 3 or 4 months before serving as subjects for studies described in this report.

The children were dwarfed in relation to standards for North American children although lengths and weights were at or above twenty-fifth percentile values of growth charts constructed from data pertaining to presumably healthy African

TABLE 1

Results of nitrogen balance studies with normal 4- to 6-month-old North American infants during the fourth through sixth days after institution of a protein-deficient diet

Subject	Birth wt	Age	Weight	Length	Caloric intake	Nitrogen balance				Urinary excretion of nitrogen		
						Intake	Excretion		Retention	Day 4	Day 5	Day 6
							Urine	Feces				
	g	*days*	*g*	*cm*	*kcal/kg/day*	*mg/kg/day*				*mg/kg/day*		
	(a) Boys; mean intake of protein 0.39 g/kg/day; prior intake of protein approximately 2.5 g/kg/day											
D.Mi.	3,450	140	5,825	62.3	118	68.9	43.8	44.6	−19.5			
G.Ch.	3,660	126	6,100	62.9	90	57.4	40.0	30.5	−13.1			
C.Ke.	3,360	141	6,000	63.5	99	70.9	41.7	42.2	−13.0			
B.Ba	3,320	127	6,170	60.5	120	63.4	37.9	41.0	−15.5			
B.Al.	2,970	134	6,800	63.1	85	57.0	39.0	30.9	−12.9			
T.In.	3,615	129	5,885	63.4	112	68.6	38.9	52.7	−23.0			
C.Sc.	2,580	129	5,635	61.4	92	64.1	40.5	31.2	− 7.6			
R.Hi.	3,070	125	7,110	62.6	110	55.2	35.2	62.9	−42.9			
Mean		131.4	6,191	62.5	103	63.2	39.6	42.0	−18.4			
	(b) Girls; mean intake of protein 0.16 g/kg/day; prior intake of protein approximately 2.5 g/kg/day											
S.We.	3,160	131	6,790	63.3	85	29.7	41.1	13.6	−25.0	56.8	29.9	36.5
T.Si.	3,870	144	6,610	64.8	73	25.6	32.4	16.8	−23.6	29.5	32.1	35.7
K.Wo.	3,380	136	7,655	62.1	57	19.6	36.4	19.7	−36.5			
K.Ir.	3,190	142	6,515	68.1	101	27.0	37.0	22.4	−32.4	49.6	34.7	26.7
M.Fo.	3,910	176	7,700	65.2	71	25.2	38.9	17.0	−30.7	32.6	34.9	49.2
D.To.	2,980	185	7,980	69.0	74	21.1	39.8	18.6	−37.3	54.9	30.3	34.1
T.Br.	3,260	187	8,100	67.4	92	29.3	32.8	32.7	−36.2	38.5	31.3	28.7
Mean		157.3	7,336	65.7	79	25.4	36.9	20.1	−31.7	43.7	32.2	35.2
	(c) Boys; mean intake of protein 0.19 g/kg/day; prior intake of protein approximately 4.5 g/kg/day											
D.Jo.	4,760	180	9,625	71.3	87	30.0	36.2	36.7	−42.9			
D.Ke.	3,310	177	7,260	67.8	146	27.8	49.0	35.1	−56.3	52.0	48.2	46.8
J.Ch.	2,940	176	7,545	66.0	117	36.9	46.5	20.8	−30.4	60.3	41.8	37.5
J.Jo.	4,370	173	8,835	71.2	95	26.1	37.4	37.2	−48.5	37.1	39.8	35.2
Mean		176.5	8,316	69.1	111	30.2	42.3	32.5	−44.5	49.8	43.3	39.8

children from the same locality.[3] At the time of study mean height-age and weight-age (both determined from Iowa Growth Chart (9)) of boys were identical (3 years 5 months) but considerably retarded in relation to the actual mean age of 5 years 4 months. Mean height-age and mean weight-age of girls were 3 years 6 months and 3 years 1 month, respectively, compared with mean actual age of 4 years 9 months.

The protein-deficient diet has been described previously (8). Caloric intake was 1500 kcal/day and intake of protein was less than 0.1 g/kg/day. Thus, for practical purposes the diet may be considered "protein-free" and this designation has been used throughout the remainder of the report.

Although most of the children received the protein-free diet on more than one occasion, 2 to 3 months of ingestion of a diet adequate in protein were nearly always interspersed between the short intervals of ingestion of the protein-free diet. Dietary intake of each child during this entire period of hospitalization was certainly superior to that of the great majority of children of similar age in the community.

PROCEDURES AND METHODS

Procedures and methods used in the metabolic balance studies with infants have been described by Fomon et al. (10). Metabolic balance studies were performed during the fourth through sixth days after institution of the protein-deficient diet.

Procedures and methods used in studies of the African children have been described by DeMaeyer and Vanderborght (8, 11). Metabolic balance studies were begun on the third day after institution of the protein-free diet and were of 3 to 5 days' duration. In 5 instances, the protein-deficient diet was administered for 14 days and 5-day metabolic balance studies were performed during the third through seventh days and the tenth through fourteenth days after institution of the protein-free diet.

RESULTS

Studies with 4- to 6-month-old infants. Results of metabolic balance studies are presented in table 1. One 3-day metabolic balance study was performed with each of nineteen 4- to 6-month-old infants during the fourth through the sixth days after institution of the protein-deficient diet (table 1). Mean caloric intake was 96 kcal/kg/day (range 57 to 146) and mean intake of protein (nitrogen × 6.25) was 0.26 g/kg/day (range 0.12 to 0.44). Nitrogen balance was negative in all instances, ranging from −7.6 to −56.3 mg/kg/day.

Data included in the present report are not suitable for demonstrating a sex-related difference in urinary or fecal excretion of nitrogen by infants if such difference exists. However, many metabolic balance studies with infants receiving various intakes of protein [4] have failed to disclose a sex-related difference in urinary or fecal excretion of nitrogen and no sex-related difference could be demonstrated in study of the African children. In this report the authors have therefore ignored the possibility that differences in data presented in the 3 parts of table 1 are influenced by the fact that boys served as subjects of studies summarized in table 1 (a and c) and girls served as subjects of studies summarized in table 1 (b).

Protein content of protein-deficient diet: Although the protein content of the protein-deficient diet was in no instance more than 0.44 g/kg/day (dietary nitrogen × 6.25), higher intakes of nitrogen generally resulted in greater urinary excretions than did lower intakes. Thus, during metabolic balance studies with 15 infants whose prior dietary histories had been similar, mean urinary excretion of nitrogen by 8 infants (table 1 (a)) receiving protein intakes of 0.34 to 0.44 g/kg/day was 39.6 mg/kg/day; during balance studies with 7 infants (table 1 (b)) receiving protein intakes of 0.12 to 0.19 g/kg/day, mean urinary excretion of nitrogen was 36.9 mg/kg/day. The difference is not statistically significant (13) at the 95% level of confidence ($t = 2.01$; $0.1 > P > 0.05$).

Fecal excretion of nitrogen was influenced to an even greater extent than was urinary excretion by the protein content of the protein-deficient diet. With mean

[3] Unpublished growth charts of E. M. DeMaeyer.

[4] Reference to a number of the studies carried out at the State University of Iowa is made in a report (12) published in 1961.

intakes of protein of 0.39 (table 1 (a)) and 0.16 g/kg/day (table 1 (b)), mean fecal excretions of nitrogen were 40.7 and 20.1 mg/kg/day, respectively. This difference is significant at the 99% level of confidence ($t = 3.7$).

Prior dietary history: An indication of the influence of prior intake of protein on urinary and fecal excretions of nitrogen is possible from comparison of data in table 1 (b) with those in table 1 (c). These 2 groups of infants received similar intakes of protein during the metabolic balance studies but the intake of protein before institution of the protein-deficient diet had been approximately 2.5 g/kg/day by one group (table 1 (b)) and 4.5 g/kg/day by the other (table 1 (c)). Mean urinary excretions of nitrogen were 36.9 and 42.3 mg/kg/day, respectively. The difference between these values is statistically significant at the 95% level of confidence ($t = 2.27$).

Mean fecal excretion of nitrogen was also greater by infants whose prior intake of protein had been greater (32.5 versus 20.1 mg/kg/day and the difference in mean fecal excretions was statistically significant at the 98% level of confidence ($t = 2.93$).

Duration of administration of protein-deficient diet: Each 24-hour collection of urine was analyzed during nine of the metabolic balance studies with infants whose intake of protein during the metabolic balance studies ranged from 0.13 to 0.23 g/kg/day. Mean urinary excretions of nitrogen by 6 infants whose prior dietary intake of protein had been 2.5 g/kg/day (table 1 (b)) were 43.7, 32.2 and 35.2 mg/kg/day, respectively, on the fourth, fifth and sixth days after institution of the protein-deficient diet. The difference in mean urinary excretion between the fourth and fifth days was statistically significant at the 95% level of confidence ($t = 2.27$). In studies with 3 infants (table 1 (c)) whose prior dietary intake of protein had been approximately 4.5 g/kg/day, urinary excretion of nitrogen was also somewhat greater on the fourth than on the fifth or sixth day after institution of the protein-deficient diet.

Studies of the infants were not suitable for determining the influence of duration of administration of the protein-deficient diet on fecal excretion of nitrogen.

Studies with African children. Sixty-five metabolic balance studies were performed with the 15 children. Data from 59 balance studies that began on the third day after institution of the protein-free diet are presented in table 2; these include results of seventeen 3-day balance studies, eighteen 4-day studies and twenty-four 5-day studies. Results of six other metabolic balance studies will also be presented (see description of duration of administration of protein-free diet).

Since intake of protein (nitrogen × 6.25) during a metabolic balance study was in each instance less than 0.1 g/kg/day, it is assumed that variations in protein intake during the balance studies were of no consequence.

Influence of sex and age: Graphic analysis (not included in this report) of data in table 2 (a) fails to demonstrate a significant trend toward increasing or decreasing values for urinary or fecal excretion of nitrogen in relation to age of the African children. Neither was it possible to demonstrate a sex-related difference. For example, mean urinary and fecal excretions during 17 studies with the 6 boys in group 2A were 48.0 and 24.4 mg/kg/day, respectively, whereas corresponding mean excretions during 19 studies with the 6 girls in group 2A were 47.6 and 21.5 mg/kg/day. It therefore seems unlikely that age or sex were significant variables affecting results of studies with the African children.

Prior dietary history: The influence of differences in intake of protein during the weeks preceding the protein-free diet may be seen by comparison of data in table 2 (a) with those in table 2 (b and c). Mean urinary excretions of nitrogen during the third through fifth day after institution of the protein-free diet (these mean values are not listed in the table) were 47.9, 51.2 and 66.8 mg/kg/day, respectively, for groups 2A, 2B and 2C.

Since daily mean urinary excretion of nitrogen was greater on the third than on the fourth through sixth day of ingesting the protein-free diet, urinary excretion of nitrogen during the latter interval provides a better estimate of minimal rates

TABLE 2

Results of nitrogen balance studies with 3- to 8-year-old African children receiving protein-free diets

Subject	Sex	Age	Prior protein intake	Weight	Height	Nitrogen balance				Urinary excretion of nitrogen				
						Intake	Excretion		Retention	Day 3	Day 4	Day 5	Day 6	Day 7
							Urine	Feces						
		year – month	*g/kg/day*	*kg*	*cm*	*mg/kg/day*				*mg/kg/day*				
					(a) Prior intake of protein 0.5 to 1.3 g/kg/day									
163	M	3–3	0.84	12.35	85.2	11	53	18	−60	45	35	60	55	71
109	M	4–7	1.04	14.09	96.4	15	44	16	−45	60	36	40	53	32
		5–0	0.95	15.10	96.5	9	51	19	−61	66	40	55	32	60
		5–3	0.68	15.38	98.3	8	57	22	−71	56	43	63	52	73
		5–6	0.95	16.09	100.0	11	45	19	−53	50	37	45	35	59
		5–9	0.97	16.79	100.7	10	53	20	−63	58	55	41	60	
		6–2	1.08	17.74	105.0	13	49	26	−62	63	48	37		
132	M	4–9	0.98	14.74	94.8	14	44	13	−43	45	39	48	52	37
		5–0	0.97	15.28	95.1	10	40	41	−71	29	37	42	47	47
154	M	5–5	1.16	13.94	90.0	12	64	31	−83	65	73	53	67	
		5–6	0.61	14.26	92.0	12	41	26	−55	50	42	36	35	
176	M	5–8	1.17	15.79	101.1	14	38	19	−43	40	34	39		
73	M	6–0	0.64	16.37	101.5	8	56	33	−81	66	57	59	56	42
		6–2	0.91	16.64	101.5	11	42	24	−55	55	38	42	40	39
		6–4	0.94	17.22	102.7	10	47	20	−57	48	48	44	48	
		6–6	0.51	16.94	104.1	11	48	47	−84	47	43	58	46	
		6–9	1.05	18.76	105.5	11	44	21	−54	52	45	33		
164	F	3–6	0.76	13.63	88.2	8	48	32	−72	59	43	40	50	
		3–9	1.01	14.67	88.7	12	32	25	−45	27	42	35	26	29
		3–11	1.00	16.08	90.8	11	44	29	−62	38	47	33	58	
		4–2	0.54	15.78	91.8	10	41	27	−58	42	51	30	39	
		4–5	1.09	18.11	93.0	12	32	25	−45	33	36	28		
147	F	4–0	0.99	14.62	92.4	11	39	37	−65	49	31	42	29	42
		4–3	0.75	14.11	93.2	9	66	22	−79	62	56	84	57	71
		4–6	1.04	14.25	93.3	13	40	21	−48	61	42	33	31	33
		4–8	1.06	15.31	95.1	11	50	21	−60	59	52	46	42	
		4–10	0.59	14.46	95.3	13	41	9	−37	51	54	32	28	
		5–2	1.18	16.33	98.4	14	56	34	−76	67	42	57		
161	F	4–11	1.03	15.76	96.5	11	43	18	−50	44	37	37	52	
		5–1	0.54	15.61	98.4	11	36	16	−41	33	37	39	33	
		5–5	1.15	16.27	100.0	13	57	17	−61	67	59	44		

TABLE 2 (*Continued*)

104	F	5–0	1.06	14.07	95.8	7	75	6	−74	71	77	79	73	73
		6–3	0.88	16.44	101.9	13	45	3	−35	54	37	54	40	40
		6–6	0.84	17.70	103.5	8	62	14	−68	61	57	71	71	48
49	F	6–5	0.87	17.28	106.7	6	46	34	−74	49	42	40	50	51
43	F	7–10	1.27	21.02	108.3	5	51	18	−64	46	37	58	49	63
Mean			0.89	15.8	97.3	10.8	47.8	22.9	−59.9	51.9	45.3	46.6	46.9	50.6
					(b) Prior intake of protein 1.4 to 1.7 g/kg/day									
109	M	6–0	1.44	17.26	103.1	9	38	22	−51	44	27	36	45	
176	M	5–6	1.60	15.75	99.4	9	48	23	−62	49	28	54	60	
73	M	6–7	1.43	17.60	105.0	8	68	25	−85	74	66	61	69	
		6–10	1.69	18.28	106.0	9	59	13	−63	70	52	55		
164	F	4–3	1.51	16.47	92.4	9	32	26	−49	26	23	33	45	
		4–7	1.60	17.77	95.0	9	48	16	−55	42	42	58		
147	F	3–9	< 1	14.10	91.5	15	56	8	−49	52	60	55	56	58
		5–0	1.57	15.70	96.0	9	39	15	−45	46	33	39	39	
161	F	5–3	1.48	16.05	98.4	9	64	29	−84	50	70	41	97	
		5–6	1.68	15.90	100.0	10	65	22	−77	68	59	69		
104	F	5–2	1.43	14.47	96.8	9	50	55	−96	41	39	75	41	52
93	M	4–2	1.41	13.81	95.0	8	62	40	−94	59	77	58	58	57
75	F	4–7	< 1	14.09	91.2	8	45	33	−70	36	41	38	66	42
		5–0	1.49	15.16	95.0	9	65	47	−103	77	50	77	64	69
		5–3	1.63	15.82	96.0	7	50	54	−97	50	47	55	42	55
Mean			1.46	15.9	97.4	9.1	52.6	28.5	−72.0	52.3	47.6	53.6	56.8	55.5
					(c) Prior intake of protein 4.5 to 6.3 g/kg/day									
109	M	6–6	4.87	17.64	107.0	11	50	40	−79	48	43	43		
176	M	6–0	5.46	15.68	104.0	13	50	29	−66	54	36	58		
73	M	7–1	4.50	18.84	107.0	11	69	36	−97	61	74	72		
164	F	4–8	4.73	17.82	95.5	11	89	16	−94	91	87	88		
147	F	5–3	5.17	15.99	99.0	10	77	26	−93	76	60	94		
		5–6	5.25	16.33	101.0	12	86	34	−108	83	84	90		
161	F	5–9	5.19	16.40	100.6	12	57	41	−86	38	68	65		
208	M	5–0	6.30	13.64	88.6	15	56	24	−65	43	45	79		
Mean			5.18	16.5	100.3	11.9	66.8	30.8	−86.0	61.8	62.1	73.6		

of excretion than does excretion during the third through sixth day. Mean urinary excretion of nitrogen during the fourth through sixth day of ingesting the protein-free diet was 46.0 for group 2A and 51.9 for group 2B. This difference is not statistically significant at the 95% level of confidence ($t = 1.90$; $0.1 > P > 0.05$).

Further subdivision of data in table 2 (a) discloses that mean urinary excretion of nitrogen during the fourth through sixth day after institution of the protein-free diet was 46.2 in 21 metabolic balance studies with 10 infants whose prior intakes of protein had ranged from 0.51 to 0.99 g/kg/day and 45.6 in 15 metabolic balance studies with 9 infants whose prior dietary intake of protein had ranged from 1.00 to 1.27 g/kg/day. It is therefore concluded that minimal rates of urinary excretion of nitrogen were achieved when prior intakes of protein varied from 0.5 to 1.3 g/kg/day.

Since data from 3-day metabolic balance studies are too few to warrant comparison of data in table 2 (a, b and c), all available balance studies have been utilized in analysis of the influence of prior dietary intake of protein on fecal excretion of nitrogen. With prior intakes of protein of 0.5 to 1.3 (table 2 (a)), 1.4 to 1.7 (table 2 (b)) and 4.5 to 6.3 g/kg/day (table 2 (c)), mean fecal excretions of nitrogen were 22.8, 28.5 and 30.8 mg/kg/day, respectively. Differences in mean fecal excretion of nitrogen were statistically significant at the 95% level of confidence between groups 2A and 2C ($t = 2.18$) but not between groups 2A and 2B ($t = 1.65$; $0.2 > P > 0.1$) or between groups 2B and 2C ($t = 0.39$; $P > 0.7$). When data from table 2(a) were further subdivided, mean fecal excretion of nitrogen was 23.6 mg/kg/day in 21 metabolic balance studies with 10 children whose prior intake of protein had ranged from 0.51 to 0.99 g/kg/day and 21.8 mg/kg/day in 15 metabolic balance studies with 9 children whose prior intakes of protein had ranged from 1.0 to 1.27 g/kg/day. It is therefore concluded that minimal fecal excretions of nitrogen were achieved when prior intakes of protein ranged from 0.5 to 1.3 g/kg/day.

Duration of administration of the protein-free diet: As shown in table 2(a) (prior intakes of protein 0.5 to 1.3 g/kg/day), mean urinary excretions of nitrogen were 51.9, 45.3, 46.6, 46.9 and 50.6 mg/kg/day on the third, fourth, fifth, sixth and seventh days, respectively, after institution of the protein-free diet. The difference in mean values for the third and fourth days was statistically significant at the 98% level of confidence ($t = 2.60$) but differences between the other days were not statistically significant (e.g., between fourth and seventh days, $t = 1.54$; $0.2 > P > 0.1$).

In 5 instances (subject 73, age 5 years 6 months;[5] 109, 4 years 7 months; 132, 4 years 9 months; 104, 6 years 3 months; 147, 3 years 9 months) the protein-free diet was administered for 14 days and two 5-day metabolic balance studies were performed: one from the third through the seventh day and one from the tenth through the fourteenth day (fig. 1) after institution of the protein-free diet. Mean urinary excretion of nitrogen on the tenth through the fourteenth day (58.6 mg/kg/day) was greater than the value of 50.2 mg/kg/day on the third through the seventh day of study ($t = 1.87$; $0.1 > P > 0.05$) or that of 48.2 mg/kg/day on the fourth through the seventh day of study ($t = 2.04$; $0.05 > P > 0.02$).

From these data it appears that minimal rates of urinary excretion of nitrogen were achieved by the fourth day after institution of the protein-free diet.

Whether rates of fecal excretion of nitrogen by children of group 2A were minimal is uncertain since all metabolic balance studies were begun on the third day after institution of the protein-free diet. Rates of fecal excretion might be less during the fourth through sixth or seventh day of ingesting the protein-free diet than during the third through fifth, third through sixth or third through seventh day. Data in table 2(a) indicate that fecal excretion of nitrogen averaged 23.7 mg/kg/day during six 3-day metabolic balance studies (third through fifth days after institution of the protein-free

[5] Data from study of subject 73 at this age were not included in table 2 since height of 99.3 cm and weight of 14.34 kg did not fulfill the previously defined criteria for adequate nutritional state.

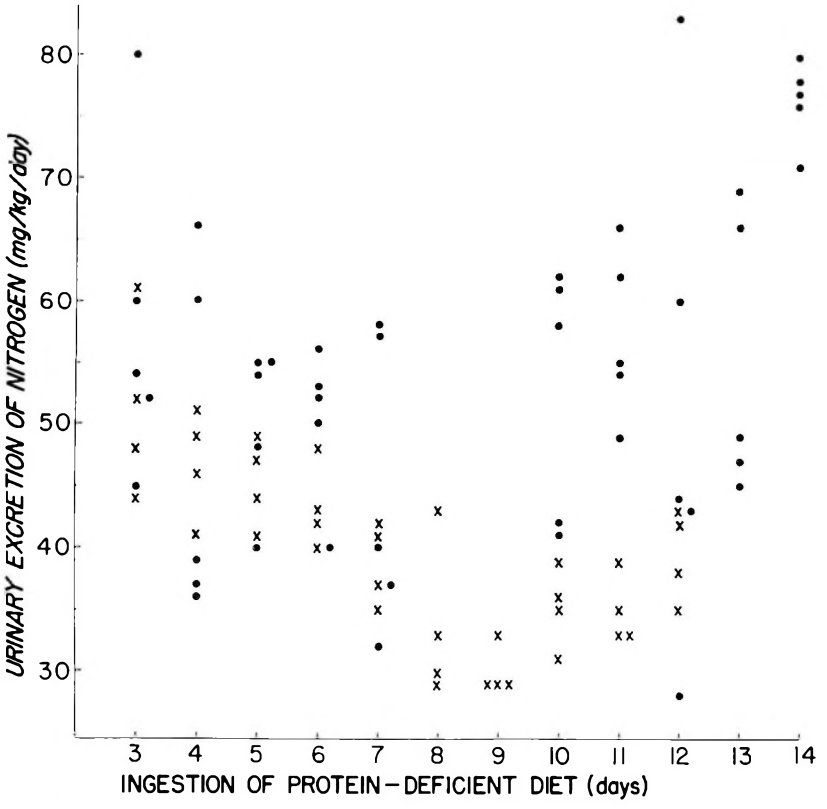

Fig. 1 Influence of duration of administration of the protein-deficient diet on urinary excretion of nitrogen by 5 African children (dots) and 4 adult subjects (x's) (6).

diet), 24.7 mg/kg/day during twelve 4-day studies (third through sixth days) and 21.4 mg/kg/day during eighteen 5-day studies (third through seventh days). These differences are not statistically significant (e.g., 4-day balances vs. 5-day balances, $t = 0.90$; $P > 0.3$).

Mean fecal excretions of nitrogen by the 5 subjects who received the protein-free diet for 14 days were 9.4 mg/kg/day (range 3 to 16) during the third through the seventh days and 20.4 mg/kg/day (range 13 to 26) during the tenth through the fourteenth days after institution of the protein-free diet. The authors have no explanation for the lesser fecal excretions of nitrogen by these 5 subjects than by most other subjects of groups 2A and 2B. Differences in mean fecal excretions of nitrogen by these subjects were significantly greater during the third through seventh day than during the tenth through fourteenth day ($t = 3.41$; $P < 0.01$).

DISCUSSION

Some portion of the nitrogen excreted in urine and feces arises from catabolic processes within the body and therefore may be considered endogenous. However, not all nitrogen liberated from tissues by catabolic processes is excreted, and the amount cycled into anabolic pathways may be surmised to be greater when the diet is deficient in protein than when adequate amounts of dietary protein are provided. Thus, urinary and fecal excretions of endogenous nitrogen are probably less by subjects receiving protein-deficient diets than by subjects receiving adequate intakes of protein.

Such considerations make it necessary to exercise caution in interpretation of values for urinary and fecal excretion of nitrogen by individuals receiving protein-

deficient diets. Furthermore, the precise conditions of study, particularly the amount of protein included in the protein-deficient diet, the duration of administration of the diet, and the prior intake of protein exert important influences on the results.

Although the African children were dwarfed in comparison with North American children of similar age, there are several reasons for considering that urinary and fecal excretions of endogenous nitrogen by these children are representative of those by healthy African children living in the same locality: all had received adequate diets for at least 3 to 4 months after recovery from illness; heights and weights of the children were at or above the 25th percentiles of growth charts constructed from data pertaining to healthy African children from the same locality; values for minimal urinary and fecal excretions of nitrogen by individual subjects studied at intervals during several months did not demonstrate a significant tendency to increase or decrease, and results of studies with subject no. 147, who had always been well, were similar to results of studies with the other subjects.

With the present lack of data on endogenous excretion of nitrogen by preschool children, it appears reasonable to conclude, tentatively, that data accumulated in study of the African children is representative of that which might be obtained from study of North American children of similar size, i.e., 3- to 4-year-olds.

Influence of duration of administration of the protein-deficient diet on urinary excretion of nitrogen by individuals of various ages may be seen from figure 1 and table 3. For each age group urinary excretion of nitrogen was somewhat greater on the third day than on the fourth through sixth day after institution of the protein-deficient diet. No consistent trend toward increasing or decreasing daily urinary excretion of nitrogen during the fourth through the sixth day was noted. During the second week of ingesting the protein-deficient diet (fig. 1), urinary excretion of nitrogen by adults was slightly less, and that by African children signifi-

TABLE 3

Influence of duration of administration of protein-deficient diet on urinary excretion of nitrogen

Reference	No. of subjects	No. balance studies	Age	Mean protein intake	Prior protein intake	Daily urinary excretion of nitrogen										
						Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
				g/kg/day	*g/kg/day*	*mg/kg/day*					*mg/kg/day*				*mg/kg/day*		
This report	6	6	4–6 mo	0.16	2.5		44	32	35								
This report	12	36 [1]	3–4 years [2]	0.07	0.5–1.3	52	45	47	47	51							
This report	5	5	3–4 years [2]	0.09	0.9–1.0	58	48	50	50	45			53	57	52	55	76
Mueller and Cox (6)	4	4	adult	0.05	1.8	51	47	45	43	38	33	30	35	35	39		
Murlin et al. (5)	48	85	adult	0.03	0.5	39											
Murlin et al. (5)	14	22	adult	0.03	0.5		32										
Hawley et al. (7)	8	8	adult	0.02	0.3		←29→										
	3	3	adult	0.02	0.3				←28→								
	10	10	adult	0.03	0.3					←30→							

[1] Data for sixth day apply to 30 studies with 11 subjects; data for seventh day apply to 18 studies with 9 subjects (table 2(a)).

[2] Height-age of African children. See text.

TABLE 4

Urinary and fecal excretion of endogenous nitrogen during the fourth through sixth days after institution of protein-deficient diet

Reference	No. of subjects	No. of balance studies	Age	Mean protein intake	Prior protein intake	Days of low protein diet	Daily excretion of nitrogen			
							Urine		Feces	
				g/kg/day	*g/kg/day*		*mg/kg mg/basal calories* [1]		*mg/kg % of total*	
(a) Prior intake of protein "low or intermediate"										
This report	7	7	4–6 months	0.16	2.5	4–6	37	0.6	20	35
This report	12	36 [2]	3–4 years [3]	0.07	0.5–1.3	4–5 or 4–6	46	0.9	23	33
This report	9	15	3–4 years [3]	0.06	1.4–1.7	4–5 or 4–6	53	1.1	29	35
Murlin et al. (5)	5	10	adult	0.03	0.5	4	31	1.4		
Hawley et al. (7)	10	18	adult	0.03	0.3	4–5 or 5–6	29	1.2	11	28
(b) Prior intake of protein "high"										
This report	4	4	4–6 months	0.16	4.0–5.0	4–6	42	0.8	33	44
This report	7	8	3–4 years [3]	0.07	4.5–6.3	4–5	68	1.4	31	31
Mueller and Cox (6)	4	4	adult	0.05	1.8	4–6	46	1.5	13	22

[1] Basal caloric expenditures were determined in the study by Murlin et al. (5). In the other studies they have been calculated on the basis of published data pertaining to infants and children (14) and adults (15).

[2] Value for fecal excretion applies to all balance studies listed in table 2(a).

[3] Height-age of African children. See text.

cantly more, than during the fourth through seventh days of ingesting the diet.

Since urinary excretion of nitrogen is relatively constant during the fourth through sixth day after institution of the protein-deficient diet, data pertaining to this interval have been used in table 4 to indicate the influence of age and prior intake of protein on urinary and fecal excretion of nitrogen. In each age group, greater prior intakes of protein result in greater urinary and fecal excretions of nitrogen. For example, mean urinary excretions of nitrogen by African children with prior intakes of protein of 0.5 to 1.3, 1.4 to 1.7 and 4.5 to 6.3 were 46, 53 and 67 mg/kg/day, respectively. Corresponding fecal excretions of nitrogen were 23, 29 and 31 mg/kg/day.

The greater urinary and fecal excretions of nitrogen reported by Mueller and Cox [6] (6) than by Murlin et al. (5) or Hawley et al. (7) (tables 3 and 4) is probably explained by greater prior intake of protein in the study by Mueller and Cox.

For purposes of classification, prior intakes of protein have been divided in table 4 into "low or intermediate" and "high." According to this arbitrary classification, an intake of protein of 1.8 g/kg/day is considered "high" for the adult and intakes of 4.0 g/kg/day or more are considered "high" for the infant and child. Other intakes are considered "low or intermediate." When prior intakes of protein had been "low or intermediate," urinary excretion of nitrogen in relation to body weight was less by adult subjects than by North American infants or African children (table 4 (a)). In relation to basal energy expenditure, urinary excretion of nitrogen increased with increasing age, the value per basal calorie for infants being one-half the corresponding value for adults.

Results of the present study are similar to those reported by Ashworth (16) and by Ashworth and Cowgill (17) from study of rats. Urinary excretion of endogenous

[6] Dr. Warren M. Cox has kindly supplied additional data about the 4 adult subjects. Heights of subjects A, B, C and D were 180, 185, 175 and 178 cm, respectively; weights at onset of study were 92.5, 71.7, 88.9, and 65.3 kg, respectively. Prior intakes of protein were approximately 1.8 g/kg/day.

nitrogen was found to be 1.0 mg/basal kcal for rats of weaning size and 1.5 mg/kcal calorie for adult rats (16). It was demonstrated that the ratio of urinary excretion of endogenous nitrogen to basal caloric expenditure increased rapidly during the period of rapid growth after weaning (17). The correlation of basal caloric expenditure with urinary excretion of endogenous nitrogen was significantly less than the correlation of either of these variables with body weight.

Fecal excretion of endogenous nitrogen by infants and children accounts for approximately one-third of total (urinary plus fecal) excretion (table 4), a slightly higher percentage than that found in studies of adults.

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Interrelation of Vitamin B_6 and Sex on Response of Rats to Hypercholesterolemic Diets [1]

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ABSTRACT When weanling rats were fed purified diets with added cholesterol and cholic acid for 20 weeks, the blood cholesterol levels of females were two to three times higher than those of males. This effect was greatest when the diet contained a severely hypercholesterolemic fat (coconut oil), and least with cottonseed oil; lard was intermediate. Intermittent vitamin B_6 deficiency eliminated the sex difference in blood cholesterol in rats fed the hypercholesterolemic diets; the values for males increased, whereas the values for females decreased to a point where the sex difference was not significant. Coconut oil in the diet, especially combined with a vitamin B_6 deficiency, caused marked liver cirrhosis, enlarged adrenals, kidney damage and nodules and hyperplasia in the stomach, as well as a pronounced increase in sudanophilic lesions in the aorta.

Rinehart and Greenberg (1) have shown that monkeys subjected to chronic dietary deprivation of vitamin B_6 develop a variety of sclerotic lesions including atherosclerosis and cirrhosis of the liver. The blood cholesterol level of the deficient animals was unaltered, although diets with added cholesterol increased the blood cholesterol values more in deficient animals than in control monkeys (2).

In rats deficient in vitamin B_6, Swell et al. (3) observed no change in blood cholesterol, nor were any lesions in the aorta noted. However, the free cholesterol content of the liver was significantly reduced. The diet used by these investigators did not contain cholesterol.

In chickens deficient in vitamin B_6, Dam et al. (4) have shown an increased level of plasma cholesterol, but not liver cholesterol. When fed diets with 1% added cholesterol the effect of vitamin B_6 deficiency was very pronounced. The aortas accumulated high levels of cholesterol and lipid but no specific lesions were noted in the 4-week experiment.

In view of these results, an attempt has been made to produce in rats severe aortic lesions, similar to those observed in monkeys, by combining an intermittent vitamin B_6 deficiency with dietary hypercholesterolemia.

MATERIAL AND METHODS

Sixty weanling rats of an Osborne-Mendel derived strain (FDA Division of Nutrition stock), divided equally as to sex, received diets containing: (in per cent) vitamin test casein,[2] 15; sucrose, 53; salt mixture,[3] 4; fat, 20; vitamin mixture, 4; cholesterol, 3; and cholic acid, 1. The vitamin mixture was compounded to furnish: (in μg/g diet) thiamine·HCl, 3; riboflavin, 4; Ca pantothenate, 15; nicotinic acid, 37; biotin, 0.15; vitamin B_{12}, 0.04; folic acid, 0.1; vitamin A acetate, 2.5; *dl*-α-tocopheryl acetate, 100; inositol, 370; and choline·HCl, 1500. The diluents for the vitamins were cellulose,[4] 3, and medium chain triglycerides from coconut oil,[5] 1. The fats were commercial food grades of coconut oil,[6] leaf lard or cottonseed oil. One-half of the animals received these diets with pyridoxine·HCl added at 3 μg/g. To keep the deficient animals alive over the 20 weeks of the experiment they were occasionally fed the added-pyridoxine diet, as necessary. The fats, coconut oil, lard, or cottonseed oil, provide a wide range of lipid unsaturation.

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[1] Presented in part at the meeting of the Federation of American Societies for Experimental Biology in Chicago, April 13, 1964.

[2] "Vitamin-Free" Test Casein, General Biochemicals Incorporated, Chagrin Falls, Ohio.

[3] Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, 24: 245; obtained from Nutritional Biochemicals Corporation, Cleveland.

[4] Cellu Flour, Chicago Dietetic Supply House, Chicago.

[5] Medium chain length fraction of coconut oil, Drew Chemical Company, New York.

[6] Commercial (i.e., unhydrogenated) coconut oil contains approximately 1.6% linoleic acid; at 20% of the diet this would supply about 0.4% of calories which should be sufficient to prevent EFA deficiency.

At the tenth and thirteenth weeks blood samples were drawn from the tail vein; at termination, blood was drawn by heart puncture. The total cholesterol was determined by the method of Abell et al. (5). Heart and aorta were removed, opened lengthwise, fixed in buffered formalin, and stained grossly with Sudan IV in propylene glycol. They were examined for the following abnormalities: sudanophilia, thickening, roughness, and non-staining granular deposits, especially at main branches and valves. Grading was on a 0 to 4 scale, separately, for the ascending and descending aortic portions. Combination of values gave a scale of 0 to 8/rat. The group score was expressed as the percentage of total possible for all rats in the group (including animals with no abnormalities). Kidney, stomach, small intestinal mucosa, adrenal, pituitary and liver were examined and retained for histologic study. Sections were stained with hematoxylin and eosin; occasional sections were stained for connective tissue (Masson), calcium (Von Kossa), and fat (frozen section oil red 0). Total liver fat (gravimetric) and lipid phosphorus of the liver were determined by standard procedures. Free cholesterol of liver was determined by the Sperry-Webb method (6).

The blood cholesterol data were subjected to analysis of variance (F test). Averages of groups are expressed as geometric means, which simplifies the statistical treatment.

RESULTS

The average weight gains of rats on the various treatments are shown in figure 1.

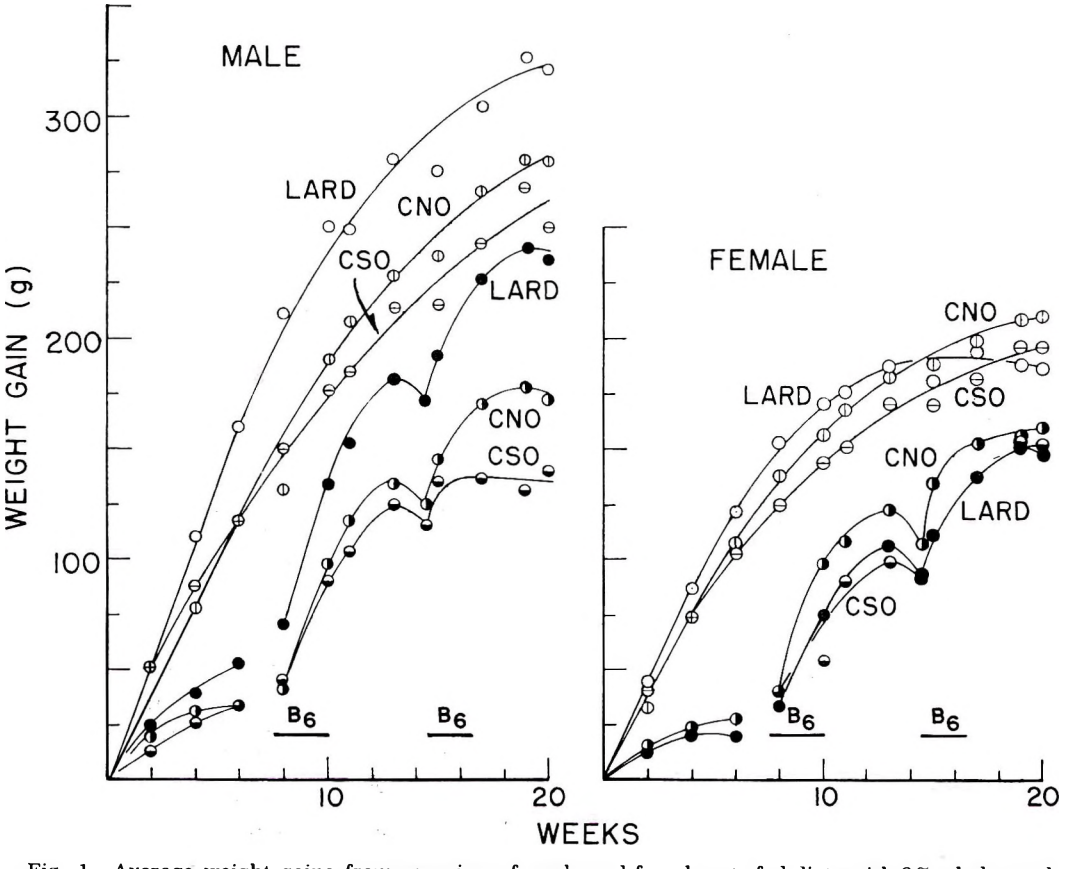

Fig. 1 Average weight gains from weaning of male and female rats fed diets with 3% cholesterol, 1% cholic acid and with ◉ or without ● vitamin B_6 supplementation. Diets: coconut oil, ◍; cottonseed oil, ⊖; and lard, ○. The periods of vitamin B_6 supplementation are indicated on the graph.

Twice during the 20 weeks of the experiment the deficient groups were fed the diets with added pyridoxine for one or two weeks; the effect on survival and weight gain was pronounced. In spite of this precautionary treatment three of the deficient rats given the coconut oil diet did not survive. The appearance and behavior of all control rats were normal, except for the oil-soaked hair of the rats receiving cottonseed oil in their diets.

Blood cholesterol. The blood total cholesterol was determined after 10, 13 and 20 weeks on experiment. The geometric means for each treatment are shown in table 1. Analysis of variance was carried out on the data for each period. Several of the differences observed in each of the periods were statistically significant at better than $P = 0.05$, as follows:

1. Female rats had significantly higher blood cholesterol than male rats in all groups of the control animals receiving vitamin B_6, regardless of type of dietary fat. However, the difference due to sex was most pronounced in the groups fed the diet with coconut oil; with this fat in the diet the sex effect was significant in the vitamin B_6-deficient groups, except at the thirteenth week.

2. The type of dietary fat had a profound effect on blood cholesterol. In all treatments and periods, rats fed the coconut oil diet had significantly higher values than rats fed either the lard or the cottonseed oil diets. Lard in the diet resulted in higher blood cholesterols as compared with cottonseed oil in the diet; however, in a number of the individual treatment groups, this difference was not statistically significant.

3. There was a significant interaction of vitamin B_6 and sex on the level of blood cholesterol (table 2). In general, the deficiency tended to minimize the sex difference in cholesterol levels. From the data in table 2 it is apparent that the blood values of the deficient males increased, whereas those of deficient females were lower, as compared with the control rats of the respective sex. Thus, at the twentieth week, for all rats, the sex-related effect in the added pyridoxine controls was a significant fourfold difference (293 vs. 1092 mg/100 ml). The deficient rats, on the other hand, showed only a small difference in mean blood cholesterol (350 vs. 470 mg/100 ml). This relation was also apparent for values for the tenth and thirteenth week. Here detailed examination shows that most of the sex-vitamin B_6 interrelation stems from the coconut oil-fed groups, where the differences are great enough to carry significance to the entire

TABLE 1

Total blood cholesterol as related to vitamin B_6 status and sex of rats raised from weaning with a diet containing 3% cholesterol, 1% cholic acid, and 20% coconut oil (CNO), lard, or cottonseed oil (CSO)

Dietary fat	Total blood cholesterol [1]						
	Males		Females		All, with vitamin B_6	All, without vitamin B_6	All rats
	With vitamin B_6	Without vitamin B_6	With vitamin B_6	Without vitamin B_6			
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
			10 Weeks				
CNO	420	687	1600	1423	819	939	867
Lard	222	233	426	350	307	285	296
CSO	110	178	337	143	181	158	169
			13 Weeks				
CNO	437	708	1346	742	767	723	748
Lard	331	299	684	414	476	352	409
CSO	220	306	303	328	254	319	284
			20 Weeks				
CNO	582	745	1492	1066	933	840	897
Lard	303	288	1630	519	640	374	490
CSO	142	211	497	313	248	263	255

[1] Values as geometric mean of 4 to 5 animals/group.

treatment, even though the values from the subgroups of lard and cottonseed oil fall short of statistical significance in some cases.

Whole blood cholesterol of our rats fed similar diets without added dietary cholesterol and cholic acid average 100 to 125 mg/100 ml with no significant sex differences.

Liver lipids. From the data in table 3 it is apparent that intermittent vitamin B_6 deficiency had no consistent effect on liver lipid composition, except to lower free cholesterol from an average of 0.55 $\pm$ 0.04 to 0.33 $\pm$ 0.02 mg/100 ml. This agrees with the observation of Swell et al. (3). The livers of all animals were fatty. The most severe damage occurred in the livers of rats fed the coconut oil diets; these livers were larger and were mottled in color, with depressed areas measuring 2- to 8-mm in diameter. Livers so damaged were tentatively classified as cirrhotic. In spite of the larger size of these livers from the rats fed coconut oil, the total fat and total cholesterol were consistently less than for rats receiving the lard or cottonseed oil diets. No effect of sex was apparent on liver composition; the liver cholesterol did not appear to vary with blood cholesterol values.

Liver pathology. Histopathologic study of the liver confirmed the presence of extensive cirrhosis and fibrotic changes in the livers from the rats fed coconut oil. Figure 2 shows some typical sections. Without cholesterol in the diet, coconut oil caused no abnormality (A). With cho-

TABLE 2

Vitamin B_6 — sex interrelation in rats fed diets with 3% cholesterol and 1% cholic acid

	Sex	Blood cholesterol [1]		
		10 weeks	13 weeks	20 weeks
		mg/100 ml	*mg/100 ml*	*mg/100 ml*
With dietary vitamin B_6	males	217	317	293
With dietary vitamin B_6	females	639	691	1092
Without dietary vitamin B_6	males	299	393	350
Without dietary vitamin B_6	females	343	434	470

[1] Values as geometric means after indicated weeks from weaning. Each group has 12 to 15 rats from the combined subgroups fed coconut oil, lard or cottonseed oil. Analysis of variance (F test) shows sex difference to be significant in the controls (added vitamin B_6), but not significant in the deficient rats.

TABLE 3

Effect of vitamin B_6, sex and type of dietary fat on liver lipids of chronic cholesterolemic rats (fed diets 20 weeks; 4 to 5 rats/group)

Sex	Dietary		Body wt	Liver wt	Liver composition				
	Fat	Vitamin B_6			Total fat	Cholesterol		Phospho-lipid	Neutral fat
						Total	Free		
			g	*% body wt*	*% fresh wt*	*% fresh wt*	*% fresh wt*	*% fresh wt*	*% fresh wt*
Males	CNO [2]	+	329	10.7	30.7	14.1	0.5	1.5	4.2
	Lard	+	374	8.1	40.2	17.5	0.6	1.5	7.8
	CSO [3]	+	305	7.6	39.5	17.6	0.4	1.5	7.2
	CNO	−	223	9.6	31.7	13.3	0.4	1.4	6.8
	Lard	−	286	8.1	41.4	17.2	0.3	1.7	9.9
	CSO	−	198	8.4	42.6	18.8	0.3	1.7	8.0
Females	CNO	+	257	8.4	35.0	15.2	0.5	1.6	6.6
	Lard	+	234	10.2	41.3	19.9	0.6	1.2	5.0
	CSO	+	242	7.7	38.3	17.0	0.7	1.7	6.5
	CNO	−	209	7.4	31.5	14.4	0.4	1.6	4.6
	Lard	−	194	7.8	36.8	17.7	0.3	1.3	4.6
	CSO	−	199	8.1	34.7	15.4	0.3	1.4	6.3

[1] By difference, assuming 18:1 as average cholesterol ester fatty acid.
[2] CNO indicates coconut oil.
[3] CSO indicates cottonsed oil.

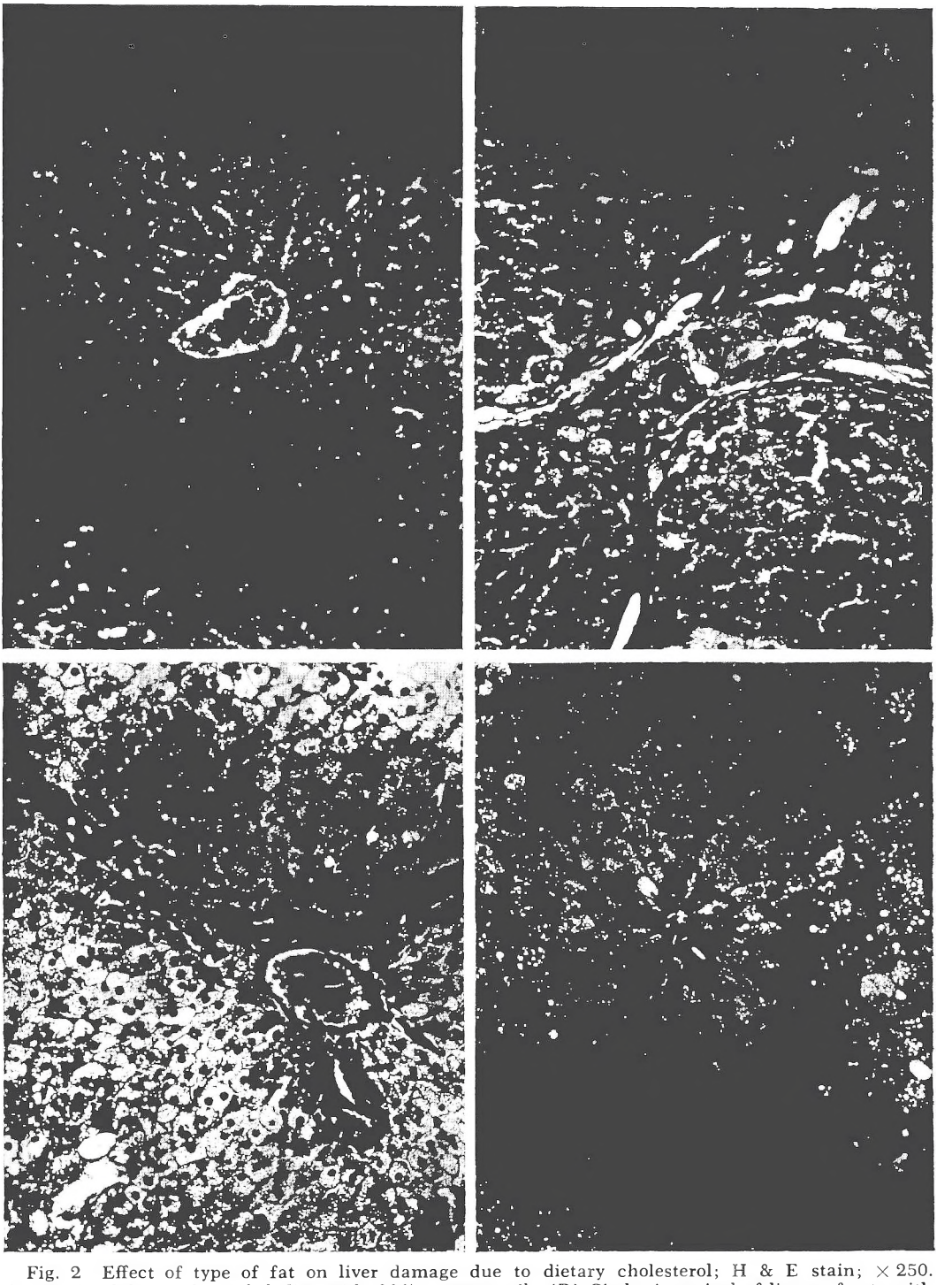

Fig. 2 Effect of type of fat on liver damage due to dietary cholesterol; H & E stain; × 250. (A) Normal liver of rat fed diet with 20% coconut oil. (B) Cirrhosis typical of livers of rats with 3% cholesterol in diet with 20% coconut oil. (C) Liver damage typical of rats fed diet with 3% cholesterol and 20% lard. (D) Fatty liver without fibrous changes typical of rats fed diet with 3% cholesterol and 20% cottonseed oil.

lesterol in the diet, the typical disruption of the structure caused by fibrous tissue invasion, typical of severe cirrhosis, occurred in all coconut oil-fed rats (B). With lard diets (and cholesterol) some inflammatory reactions and bile duct proliferation occurred, but no cirrhosis (C). With cottonseed oil (and cholesterol) diets (D) there was little damage or disruption of cellular pattern, except, of course, that all cells were fat-laden.

The vitamin B_6 deficiency appeared to aggravate the liver pathology, but this could not be quantitized. The bile duct proliferation and fibrous changes appeared to be more severe for all types of fat in the diet of the deficient rats.

Aortic lesions. The sudanophilic lesions of the aorta and heart valves were evaluated and scored (table 4). Cottonseed oil in the diet afforded considerable protection; only 21% of these rats (4/19) showed lesions, and these were of low severity. The incidence of lesions in the rats fed the lard diets was 79% (15/19), and their severity was considerably greater. In rats fed the coconut oil diets the incidence was 88%, and the degree of severity was high.

An interrelation of sex and aortic lesions was evident; females fed the coconut oil diets were evaluated as having about twice the severity score of males. Similarly, the effect of vitamin B_6 was observed, in that deficient rats had greater incidence (19/26 or 73%) as compared with the control rats (15/29 or 52%), and a greater severity (22% as compared with 9%) for all rats of the respective groups. A combination of the vitamin B_6 deficiency and a coconut oil diet, with cholesterol, was the most damaging to the aortae, particularly in the female.

Histologically, arotae from the more severely affected animals showed an occasional fibrous swelling. Considerable thickening of the walls was noted near the heart and valve area.

Other pathology. The hearts from rats fed the coconut oil diets had extensive fibrosis around the valves. Some myocardial degeneration was noted most frequently in vitamin B_6-deficient animals fed the coconut oil diets, similar to the lesions reported by Seronde (7). The coronary vessels as well as other arteries throughout the body had large vacuoles, in the intimal cells, which were presumably lipid; however, no plaques or emboli were noted.

Kidneys had a granular surface in several cases. On histologic study, a number of these showed severe damage, characterized by calcium deposits, glomerular degeneration, congestion and casts in tubules, collapsed tubules, and densely cellular portions. This damage was scattered through all groups, but was most severe in vitamin B_6-deficient females fed coconut oil. Rats fed these diets without cholesterol had normal kidneys.

Adrenal glands from the coconut oil groups were enlarged, but not hemorrhagic. The cells were large and uniformly

TABLE 4

Aortic lesion incidence and severity in rats fed high cholesterol diet for 20 weeks, as influenced by vitamin B_6, sex and type of dietary fat

Dietary fat	Males		Females		All, with vitamin B_6	All, without vitamin B_6	All rats
	With vitamin B_6	Without vitamin B_6	With vitamin B_6	Without vitamin B_6			
	Incidence, number with lesions/number in group						
CNO [1]	3/5	4/4	5/5	3/3	8/10	7/7	15/17
Lard	3/5	4/5	3/4	5/5	6/9	9/10	15/19
CSO [2]	1/5	2/4	0/5	1/5	1/10	3/9	4/19
	Severity, % of maximum score/group						
CNO	7	34	15	58	11	46	28
Lard	12	15	16	20	14	18	16
CSO	2	5	0	1	1	3	2

[1] CNO indicates coconut oil
[2] CSO indicates cottonseed oil

foamy in appearance. The borders between zones in the cortex, and between the cortex and the medulla were completely absent.

The stomach showed a number of lesions, including hyperplasia of the forestomach, nodules in the fundus, and in a few cases petechial hemorrhages in the glandular portion. These lesions were not related to dietary fat but were related to sex and vitamin B_6: Among females the ulcer incidence was 9 out of 12 deficient rats, and 5 out of 13 control rats; among males the incidence was only 3 out of 13 deficient rats, and one out of 15 control rats.

DISCUSSION

Higher blood cholesterol in female rats, compared with male rats, fed cholesterol-containing diets has been noted by Okey et al. (8, 9) and by Cuthbertson et al. (10). If the data of Van Handel and Zilversmit (11) for rabbit serum cholesterol is rearranged by sex, the value for females is twice that for male rabbits. Our data amply confirm these earlier observations.

An intermittent deficiency of vitamin B_6 tended to eliminate the sex effect on blood cholesterol level in rats fed hypercholesterolemic diets. The male rat deficient in this vitamin had a higher value than normal; the female rats had a lower value. The somatic or hormonal factor(s) at work to effect the sex difference in handling dietary cholesterol must require vitamin B_6 for activity. It is obvious from the data that this factor does not relate uniquely to maleness or to femaleness.

Dietary cholesterol caused extensive damage to liver, kidney, adrenals, stomach, and the vascular system. In most instances the tissue damage appeared to correlate with blood cholesterol levels; an exception was noted in females deficient in vitamin B_6 receiving the coconut oil diet, where the blood cholesterol was lower but greater severity of lesions occurred. The diet with coconut oil promoted very high blood cholesterol values, and also resulted in severe fibrotic cirrhosis of the liver with extensive bile duct proliferation. In rats fed the diets with lard, or especially cottonseed oil, the livers were fatty, but with little or no cirrhosis. The lower total fat and cholesterol in livers of the coconut oil groups probably reflected the cirrhotic condition of this organ. Chronic vitamin B_6 deficiency had no effect on liver lipid composition, and its effect, if any, on liver damage could not be quantitized.

The chronic vitamin B_6 deficiency increased the severity of sudanophilic lesions of the aortae in the group with the highest blood cholesterol, namely, female rats fed the coconut oil diet. However, with lard or cottonseed oil in the diet, the vitamin B_6 deficiency did not promote aortic lesions.

ACKNOWLEDGMENTS

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Mineral Balance Studies with the Baby Pig: Effects of Dietary Vitamin D_2 Level upon Calcium, Phosphorus and Magnesium Balance [1,2]

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ABSTRACT Calcium, phosphorus and magnesium balance studies were conducted with baby pigs receiving a purified casein-glucose diet containing levels of vitamin D_2 of zero, 100, 500 and 1000 IU/kg. Pigs receiving no dietary vitamin D_2 excreted an excessive amount of fecal Ca, P and Mg but a reduced amount of urinary Ca and Mg. Retention of Ca, P and Mg was greatly reduced in vitamin D_2-deficient pigs. A dietary vitamin D_2 level of 100 IU/kg produced a normal Ca, P and Mg balance which was not increased by higher levels of dietary vitamin D_2. Increasing dietary Mg in one trial increased Mg retention in pigs receiving vitamin D_2 but did not affect Ca and P balance.

The influence of vitamin D upon calcium and phosphorus balance has been known for many years (1, 2). It is only in recent years that the influence of vitamin D upon other dietary mineral elements has been demonstrated (3–7). Although a number of mineral balance studies have been conducted with the young pig (8–14) only the study of Whiting and Bezeau (11) has dealt with the effect of vitamin D upon mineral utilization.

The present investigation was undertaken to study the effects of dietary vitamin D_2 level upon calcium, phosphorus and magnesium utilization by the baby pig as determined by mineral balance trials and to provide supplementary information toward a more accurate determination of the dietary vitamin D_2 requirement of the baby pig (15).

MATERIALS AND METHODS

Baby pigs used in this study were from 3 trials conducted to determine their vitamin D_2 requirement (15) with purified casein-glucose diets. Calcium, phosphorus and magnesium balance studies were conducted with pigs receiving zero, 100, 500 or 1000 IU of vitamin D_2/kg in the first 2 trials and zero, and 500 IU of vitamin D_2 with or without additional dietary magnesium in the third trial. Dietary levels of Ca, P and Mg were 0.8%, 0.6% and 350 ppm, respectively, in all diets except in the third trial in which two of the diets contained 750 ppm of Mg. Dietary Ca was supplied by $CaCO_3$ and $CaHPO_4 \cdot 2H_2O$. Dietary P was supplied by $CaHPO_4 \cdot 2H_2O$ and by casein with casein supplying 0.14% of P in all diets. All of the dietary Mg was supplied by $MgSO_4 \cdot 7H_2O$ with the exception of 25 ppm which was present in the other dietary ingredients.

Balance studies were conducted when the pigs were 5 or 6 weeks of age. Methods of performing the balance trials were similar to those described previously (14). Two 3-day collections with controlled intake were made on most of the animals. Analyses of food, fecal and urine Ca, P and Mg were by appropriate application of the methods of Mori (16), Gomorri (17) and Lindstrom and Diehl (18), respectively, with 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) serving as the titrant in both the Ca and Mg determinations. Data were examined by analysis of variance. Statistical significance of treatment differences was determined by the multiple range test of Duncan (19).

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[2] Presented in part before the meeting of the American Institute of Nutrition, April, 1963 (Federation Proc., 22: 491, 1963, abstract).

TABLE 1

Daily calcium, phosphorus and magnesium excretion and retention as affected by dietary vitamin D_2 level (trials 1 and 2)

	Vitamin D_2, IU/kg			
	0	100	500	1000
No. of collections	8	12	8	12
Daily food intake, g	383 ± 38 [1]	468 ± 32	514 ± 38[a]	456 ± 33
Ca balance				
Daily Ca intake, g	3.07 ± 0.31	3.74 ± 0.26	4.11 ± 0.31[a]	3.64 ± 0.26
Daily fecal Ca, g	1.48 ± 0.26[bb,c]	0.87 ± 0.09	1.05 ± 0.12	0.80 ± 0.07
Daily urinary Ca, g	0.01 ± 0.00	0.03 ± 0.01	0.05 ± 0.01[aa]	0.05 ± 0.01[aa]
Daily Ca retention, g	1.58 ± 0.18	2.84 ± 0.21[aa]	3.01 ± 0.23[aa]	2.79 ± 0.21[aa]
Ca retention, %	52 ± 5	76 ± 2[aa]	74 ± 2[aa]	76 ± 2[aa]
P balance				
Daily P intake, g	2.30 ± 0.23	2.81 ± 0.19	3.09 ± 0.23[a]	2.74 ± 0.20
Daily fecal P, g	0.90 ± 0.05[aa,b]	0.59 ± 0.07	0.76 ± 0.08	0.51 ± 0.05
Daily urinary P, g	0.46 ± 0.08	0.36 ± 0.04	0.37 ± 0.05	0.49 ± 0.06
Daily P retention, g	0.94 ± 0.12	1.86 ± 0.13[aa]	1.96 ± 0.14[aa]	1.74 ± 0.15[aa]
P retention, %	41 ± 6	66 ± 2[aa]	64 ± 1[aa]	64 ± 3[aa]
Mg balance				
Daily Mg intake, mg	134 ± 13	164 ± 11	180 ± 13[a]	160 ± 11
Daily fecal Mg, mg	83 ± 11[cc]	39 ± 7	52 ± 6	36 ± 6
Daily urinary Mg, mg	3 ± 1	16 ± 4[a]	21 ± 2[aa,b]	12 ± 4[a]
Daily Mg retention, mg	48 ± 10	109 ± 10[aa]	107 ± 13[aa]	112 ± 8[aa]
Mg retention, %	36 ± 6	67 ± 5[aa]	59 ± 3[aa]	70 ± 5[aa]

[1] Mean ± SE.
[a] Significantly greater than least value ($P < 0.05$); [aa] ($P < 0.01$).
[b] Significantly greater than least two values ($P < 0.05$); [bb] ($P < 0.01$).
[c] Significantly greater than least three values ($P < 0.05$); [cc] ($P < 0.01$).

RESULTS AND DISCUSSION

Data from the first 2 trials were combined and the results are presented in table 1. All of the pigs that received no dietary vitamin D in these trials exhibited characteristic symptoms of rickets. Food intake and consequent mineral element intakes were somewhat reduced during the balance studies in these pigs. Excessive amounts of fecal Ca, P and Mg were excreted by pigs consuming no dietary vitamin D. Excretion of Ca and Mg but not P in urine was greatly reduced in the vitamin D-deficient animals. All pigs receiving 100 IU or more of dietary vitamin D_2/kg consumed, excreted and retained relatively uniform amounts of the 3 dietary elements. Dietary intake was somewhat less in these animals than in our earlier studies (13, 14) with pigs of the same age, but percentages of Ca and P retention were very similar. Percentage utilization of Ca and P were somewhat less than that reported by Lenkeit and Freese (10) but greater than that reported by Ludvigsen and Thorbek (8). Magnesium intake was less in the present study than that reported by Lenkeit and Freese (10) because of lower dietary Mg concentration, yet the percentage Mg retention was greater.

Whiting and Bezeau (11) observed little effect of the addition of 800 IU of vitamin D/454 g to their basal diet upon Ca and P utilization. Furthermore, in their study, this level of vitamin D had a depressing effect upon dietary zinc utilization. In the present study vitamin D deficiency produced a significant depression in Ca, P and Mg retention which is well illustrated by the graphs in figures 1, 2 and 3. Perhaps the most interesting result observed is the apparent positive effect of dietary vitamin D upon Mg absorption and retention (fig. 3). Earlier studies have suggested limited effects of dietary vitamin D upon Mg absorption in the rat (3) and the chick (4). Doubtless, this accounts for the vitamin D-sparing effect of supplemental dietary Mg observed in rats (20) and dairy cattle (21). This is further substantiated by the positive effect of dietary vitamin D upon serum and bone Mg concentration

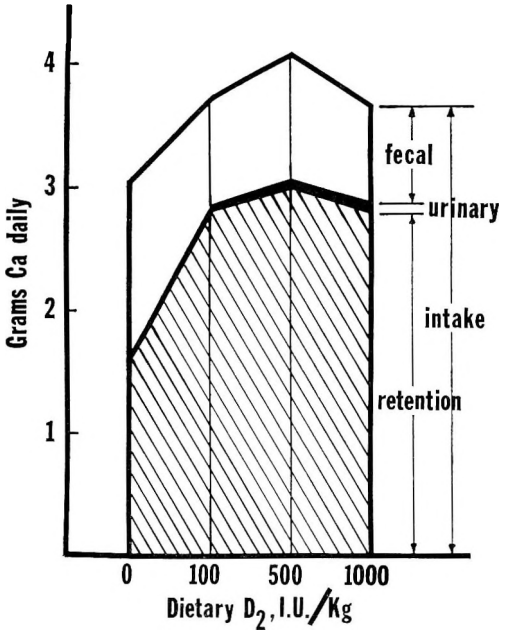

Fig. 1 Calcium balance as affected by level of dietary vitamin D_2.

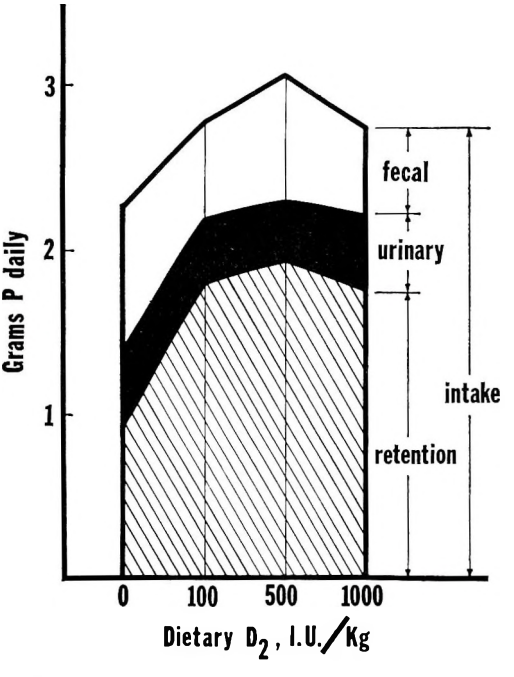

Fig. 2 Phosphorus balance as affected by level of dietary vitamin D_2.

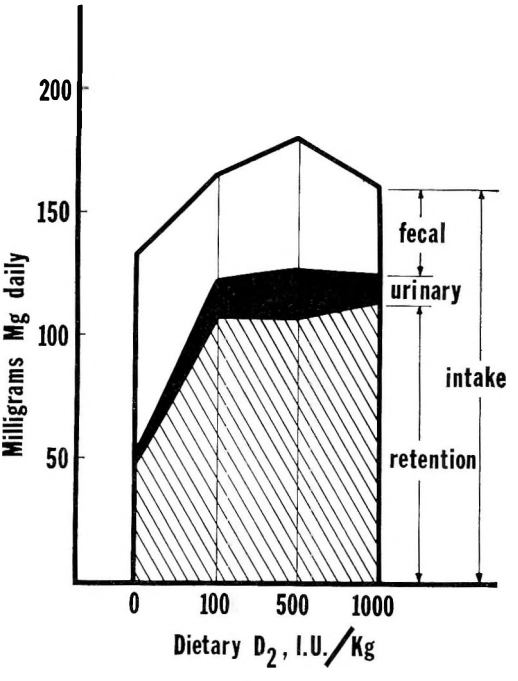

Fig. 3 Magnesium balance as affected by level of dietary vitamin D_2.

(15) and the prevention of the stepping syndrome (15, 22).

Results of trial 3 are presented in table 2. In this trial 75% of the pigs receiving no vitamin D with 350 ppm of dietary Mg died after exhibiting symptoms of Mg deficiency including the stepping syndrome and tetany. Pigs receiving no vitamin D with 750 ppm of dietary Mg did not show these symptoms but did exhibit evidence of advanced rickets (15). Voluntary food intake became considerably reduced in these animals necessitating the reduced controlled intakes for these animals during the adjustment and collection periods.

Data in table 2 indicate that dietary vitamin D has a much greater influence upon the absorption and retention of Ca, P and Mg than does dietary Mg. Increasing dietary levels of Mg above the requirement has resulted in greater Mg retention in young pigs receiving complete diets (9, 23) as observed in this study but does not affect Ca or P balance (24).

Results of the balance studies clearly indicate the necessity for vitamin D by the baby pig for adequate balance of die-

TABLE 2

Daily calcium, phosphorus and magnesium excretion and retention as affected by dietary vitamin D_2 and magnesium level (trial 3)

Dietary vitamin D_2, IU/kg	0	0	500	500
Dietary Mg, ppm	350	750	350	750
No. of collections	2	6	6	7
Daily food intake, g	300 ± 0 [1]	225 ± 0	412 ± 18^{bb}	396 ± 14^{bb}
Ca balance				
Daily Ca intake, g	2.40 ± 0.00	1.83 ± 0.00	3.30 ± 0.14^{bb}	3.17 ± 0.11^{bb}
Daily fecal Ca, g	1.10 ± 0.12^{bb}	1.17 ± 0.06^{bb}	0.58 ± 0.05	0.39 ± 0.11
Daily urinary Ca, g	0.01 ± 0.00	0.01 ± 0.00	0.07 ± 0.01^{bb}	0.07 ± 0.02^{bb}
Daily Ca retention, g	1.29 ± 0.12	0.65 ± 0.06	2.65 ± 0.12^{bb}	2.71 ± 0.10^{bb}
Ca retention, %	54 ± 5	36 ± 3	80 ± 2^{bb}	85 ± 2^{bb}
P balance				
Daily P intake, g	1.80 ± 0.01	1.35 ± 0.00	2.47 ± 0.10^{bb}	2.38 ± 0.08^{bb}
Daily fecal P, g	0.57 ± 0.06^{aa,b}	0.59 ± 0.04^{aa,b}	0.42 ± 0.04	0.27 ± 0.03
Daily urinary P, g	0.27 ± 0.02	0.27 ± 0.01	0.24 ± 0.02	0.31 ± 0.02
Daily P retention, g	0.96 ± 0.04^{aa}	0.49 ± 0.04	1.81 ± 0.10^{bb}	1.80 ± 0.06^{bb}
P retention, %	53 ± 1^{aa}	36 ± 1	73 ± 1^{bb}	76 ± 1^{bb}
Mg balance				
Daily Mg intake, mg	105 ± 0	169 ± 0^{aa}	144 ± 6^{a}	297 ± 10^{cc}
Daily fecal Mg, mg	69 ± 4	120 ± 7^{bb}	59 ± 3	143 ± 11^{bb,c}
Daily urinary Mg, mg	11 ± 2	21 ± 1	25 ± 3	63 ± 10^{cc}
Daily Mg retention, mg	25 ± 2	28 ± 7	60 ± 5	91 ± 15^{bb}
Mg retention, %	24 ± 1	17 ± 1	42 ± 1^{aa,b}	31 ± 2

[1] Mean + SE
[a] Significantly greater than least value ($P < 0.05$); [aa] ($P < 0.01$).
[b] Significantly greater than least two values ($P < 0.05$); [bb] ($P < 0.01$).
[c] Significantly greater than least three values ($P < 0.05$); [cc] ($P < 0.01$).

tary Ca, P and Mg. The data do not indicate a higher dietary vitamin D_2 requirement than that of 100 IU/kg determined by other criteria (15).

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Effect on Nitrogen Retention of Men of Varying the Total Dietary Nitrogen with Essential Amino Acid Intake Kept Constant [1]

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ABSTRACT Nitrogen balances of 6 young men on 3 different levels of total nitrogen intake but with identical intakes of purified essential amino acids plus cystine were determined. The essential amino acids plus cystine were given in the same amounts as found in 20 g egg protein. To bring the total nitrogen to the desired level, a solution of diammonium citrate and glycine was used in isonitrogenous amounts. The results indicated that 4 out of 6 subjects showed decreases in nitrogen loss as the daily nitrogen intake was increased from 4.0 to 6.0 g. All 6 subjects showed improved nitrogen retention when the total dietary nitrogen was increased to 8.0 g.

During human metabolism studies involving determination of the requirements of the essential amino acids, various investigators have used levels of total nitrogen intake ranging from 6.2 to 11.0 g nitrogen per subject per day (1–15).

Rose and Wixom (16), Swendseid et al. (17), and Tolbert and Watts (18) reported that subjects fed constant amounts of the essential amino acids had similar nitrogen balances when the total nitrogen intake was varied from 3.5 to 12 g of nitrogen per subject per day. However, Shortridge et al.,[3] Linkswiler et al. (19–20), and Clark et al. (21) observed that subjects showed better nitrogen retention with medium or high intakes of total nitrogen (8.0 to 12.0 g nitrogen daily) than with lower intakes (4.0 to 6.0 g nitrogen daily) when the intake of the essential amino acids was kept constant. Conversely, results of Tuttle et al. (22) indicated that older men fed constant amounts of the essential amino acids retained more nitrogen with an intake of 7.0 g of nitrogen than they did with 15.0 g.

The conflicting results cited above indicate a need for further investigation. The present study was designed to study the effect of progressively increasing the level of total dietary nitrogen of young men from 4.0 to 6.0 to 8.0 g/day while the intake of the essential amino acids was kept constant.

EXPERIMENTAL

The project consisted of a preliminary period of 4 days and 3 experimental periods of 12 days each.

During the preliminary period, the nitrogen intake per subject per day was 0.79 g provided by the foods in the basal diet (table 1). The purposes of this period were to provide for nitrogen depletion, to introduce the subjects to their duties and responsibilities in a research project of this type, and to accustom them to the experimental diet. The caloric intake for each subject was adjusted as needed to maintain a constant body weight by varying the intake of butter (varied among the subjects but held at a constant level for each individual after the preliminary period), certain carbonated beverages, sucrose, and hard candy.

During the experimental periods the total nitrogen intakes per subject were 4.0, 6.0, and 8.0 g, respectively. A crystalline

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[3] Shortridge, L., K. C. Chao, C. V. Kies and M. S. Reynolds 1961 Effect of source of nitrogen on utilization in adult man. Federation Proc., 20: 454 (abstract).

TABLE 1

Daily food intake during the 3 experimental periods

Food	Amount/ subject/day
	g
Applesauce	200
Peaches	100
Pears	100
Orange juice	300
Tomatoes	100
Lettuce	25
Pineapple	25
Bananas	25
Tea or coffee	varied [1]
Sugar	varied
Butter	varied [1]
Soft drink, jelly, hard candy	varied
Cornstarch wafers	1 recipe
Vitamin supplement (every other day) [2]	
Mineral supplement (daily) [3]	
Purifed essential amino acids and cystine	as in 20 g of egg protein [4]
Diammonium citrate and glycine	varied [5]

[1] The intake of tea, coffee, and butter varied among the subjects but was constant for any one subject.

[2] The vitamin supplement supplied every other day: (in milligrams) choline dehydrogen citrate, 180; thiamine·HCl, 3; niacinamide, 9; folic acid, 0.6; biotin, 0.15; vitamin B_{12}, 0.0001; D-pantothenyl alcohol, 4.5; pyridoxine·HCl, 3; riboflavin, 3; and vitamin A, 4500 USP units.

[3] The mineral supplement supplied daily: (in grams) $CaCO_3$, 1.326; KH_2PO_4, 1.307; $MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$, 0.800; $FeC_6H_5O_7 \cdot H_2O$, 0.0948; KI, 0.0078; $ZnCl_2$, 0.0019; $MnCl_2 \cdot 4H_2O$, 0.0072; and $CuSO_4 \cdot 5H_2O$, 0.0078. In addition, 7.6 g of a commercial baking powder (Calumet, General Foods Corp., White Plains, N. Y.) were used in the preparation of the cornstarch wafers.

[4] The purified amino acid mixture supplied: (in grams) L-isoleucine, 1.328; L-leucine, 1.760; L-lysine, 1.280; L-methionine, 0.627; L-phenylalanine, 1.155; L-threonine, 0.995; L-tryptophan, 0.330; L-valine, 1.484; and L-cystine, 0.467.

[5] Diammonium citrate and glycine intake was varied during the 3 experimental periods so that the total nitrogen intake was 4.0, 6.0, or 8.0 g/day, respectively.

amino acid mixture using the essential L-amino acids plus cystine as found in 20 g of egg protein provided 1.12 g of nitrogen per subject per day (table 1). Other sources of nitrogen intake were the basal diet which was constant for all periods and a solution of diammonium citrate and glycine in isonitrogenous amounts to adjust the nitrogen intake to that required by the experimental period involved. Details regarding the methods of preparation and administration of the amino acids and foods were presented in an earlier paper (15).

Six young men (table 2) were subjects for the study. All were students who carried out their usual academic pursuits during the course of the project. Physical examinations by a physician of the University Hospitals attested to the fact that all were in good health. During the experiment, the subjects lived in private homes or dormitories, but ate all of their meals in the metabolism unit of the School of Home Economics.

Nitrogen determinations by the boric acid modification of the Kjeldahl method (23) were made on the samples of foods, amino acids, diammonium citrate plus glycine solutions, urine, and feces. These materials were collected and prepared for analysis according to the methods described previously (15). Daily urinary creatinine excretions were determined using the procedure described by Peters (24).

RESULTS AND DISCUSSION

The average nitrogen balances of the individual subjects for the last 4 days of each period are shown in figure 1.

All subjects lost nitrogen at the 4.0- and 6.0-g intakes of total nitrogen, but four out of six showed less nitrogen loss with the 6.0-g nitrogen intake than with the 4.0-g level. The 2 subjects who did not show an improvement complained of respiratory infections during the final part

TABLE 2

Age, height, weight and caloric intakes of subjects

Subject	Age	Weight		Height	Avg daily caloric intake/kg body wt/day		
		Beginning	Final		Period 1	Period 2	Period 3
	years	*kg*	*kg*	*cm*	*kcal*	*kcal*	*kcal*
TM	22	73.3	73.8	179	45	45	45
RDL	23	72.0	71.6	174	49	50	50
DR	22	83.4	82.0	182	41	42	42
FE	25	70.5	70.2	179	40	40	41
HF	22	69.5	70.0	180	48	49	49
HK	22	76.8	76.8	178	47	48	48

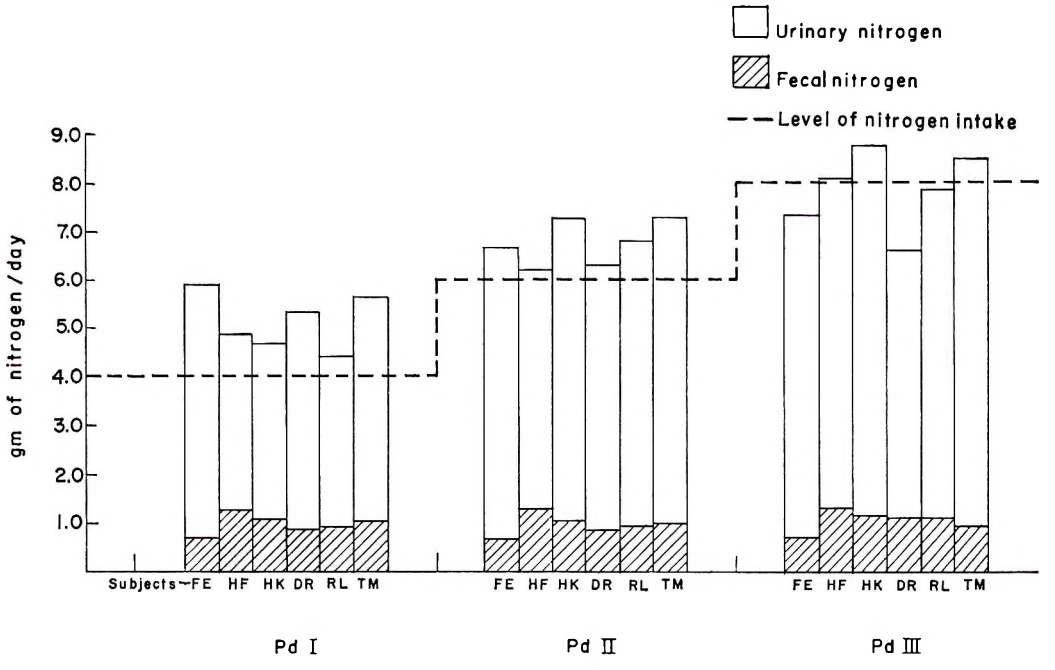

Fig. 1 Nitrogen elimination at various levels of total dietary nitrogen with intakes of essential amino acids kept constant.

of the 6.0-g total nitrogen intake period which may explain the decrease in nitrogen retention. All 6 subjects demonstrated improved nitrogen retention with the 8.0-g intake as compared with the lower intakes of total nitrogen.

The amino acid mixture used in this experiment met the daily minimum requirements of Rose (2) for all the essential amino acids except methionine; however, cystine was added in sufficient amounts so that the total sulfur-containing amino acid content exceeded the Rose minimum requirement of 1.01 g (25). The results of Rose and Wixom (16) indicated that the minimum requirements of total nitrogen when "safe" levels of the essential amino acids were given was about 3.5 g, an amount exceeded in all 3 experimental periods of the present study. However, the "safe" levels of the essential amino acids fed by Rose furnished a larger amount of nitrogen from the essential amino acids than was furnished in this study. The results of Shortridge et al.[3] and of Linkswiler et al. (19–20) were similiar to those in the present investigation, since in all 3 studies all subjects showed better nitrogen retention with higher than with lower nitrogen intakes.

However, Tuttle et al. (22) reported that subjects showed greater nitrogen retention when the total nitrogen intake was 7.0 g than when it was 15.0 g when the essential amino acid content of the diet was held constant. A synthetic mixture containing the essential amino acids as found in 300 g of whole egg with glycine or diammonium citrate and glycine to bring the total nitrogen intake to the desired level was used by Tuttle, whereas in the current project the synthetic mixture used contained the essential amino acids as found in 156 g of whole egg with diammonium citrate and glycine added in isonitrogenous amounts. The men who served as subjects in the study reported by Tuttle ranged in age from 50 to 70 years and in the present study from 20 to 24 years. Tuttle fed the subjects a diet of natural food containing either 7.0 or 15.0 g of nitrogen for at least 15 days, after which the semi-synthetic diet was given for only 5 or 6 days. In many studies con-

ducted in this laboratory, it has been found that subjects show an initial decrease in nitrogen retention when the diet is changed from one of natural foods to one semisynthetic in nature even when the nitrogen intake remains the same (14–15). Generally more than 4 days are required for subjects to regain nitrogen equilibrium after this change even when a 3- or 4-day transition period is included. Therefore, it appears possible that the 5-day experimental periods used by Tuttle were not long enough to establish a stabilized output following the diet of natural food. Additional evidence supporting this view is that positive nitrogen balances have been achieved by subjects receiving high intakes of nitrogen with similar intakes of the essential amino acids. Rose and Wixom (16) reported that subjects were in nitrogen balance when the total dietary nitrogen was 10.0 g and the essential amino acids were fed at the "safe" level. Jones et al. (15) and Reynolds et al. (14) observed that subjects achieved nitrogen balance with a total nitrogen intake of 10 to 11 g when the essential amino acids were fed in amounts found in 150 g of whole egg. Tolbert and Watts (18) also reported that young women showed greater nitrogen retention with an intake of 15 g of nitrogen daily than with a 10-g intake with constant dietary essential amino acids, although no significant difference in the nitrogen retention of these subjects was observed between a total nitrogen intake of 5.0 and the 10.0 g. Data presented by Clark et al. (21) were supportive to those of the current project.

The results of the present study suggest that the requirements of the essential amino acids for the maintenance of nitrogen balance may be influenced by the level of total nitrogen in the diet.

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Metabolic Patterns in Preadolescent Children

XII. EFFECT OF AMOUNT AND SOURCE OF DIETARY PROTEIN ON ABSORPTION OF IRON [1,2]

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ABSTRACT As one segment of a series of metabolic studies with 36 healthy preadolescent girls, the iron content of the diets and feces was determined. Iron absorption was not significantly influenced by protein, riboflavin or niacin content of the diet; however, in one study iron absorption was significantly increased when the daily protein intake was reduced by removal of all milk from the diet. A dietary allowance of at least 12 mg of iron/day is advocated for 7- to 9-year-old girls.

Recent studies (1) have demonstrated that when rats are fed diets containing less than 15% of protein, iron absorption is impaired. Reports on the influence of protein intake on iron absorption by healthy children were not found in the literature; however, Lahey et al. (2) concluded that low serum iron concentrations in children suffering from kwashiorkor were not due to deficient iron intakes but were secondary effects of inadequate protein intake.

A series of studies was conducted to investigate the effects of quantity and source of dietary protein on metabolic patterns of various nutrients in 7- to 9-year-old- girls fed controlled diets. This report presents data from these studies on intake and excretion of iron by 36 preadolescent girls fed diets supplying 6.8 to 12.3 mg of iron and 18 to 88 g of protein daily.

PLAN OF METABOLIC STUDIES AND EXPERIMENTAL PROCEDURE

The descriptive details for these studies, including objectives, general plan and organization, method used in collection and distribution of samples, dietary regimens, and characteristics of the subjects, have been published (3, 4).

The studies reported here include data for subjects maintained on controlled dietary regimens for 14 four-day periods in 1956, 8 six-day periods in 1958, and 7 six-day periods in 1962. The iron content of food and feces was determined in alternate 4-day periods in 1956 and in all periods in 1958 and 1962.

The primary nutrient variables were protein and energy in 1956; protein in 1958; and protein, riboflavin, and niacin in 1962 (3, 4). In 1956, the smaller girls were fed diet no. 2 or no. 3, supplying about 1950 kcal and the larger girls were fed diet no. 4 or no. 5, furnishing about 2400 kcal/day so that energy intake per unit of body weight would be similar for each group. Diets 9, 10, 11, and 12 in the 1962 study supplied the following calculated daily intakes of riboflavin and niacin: (in mg) 0.6, 15.2; 0.4, 4.9; 2.2, 20.4; and 2.0, 10.1; respectively. The diets were planned to meet the recommended allowances for other nutrients (5). In 1956 and 1958 dietary protein was supplied by foods of both plant and animal origin, but in 1962 protein was supplied entirely by foods of plant origin. The chief sources of protein in 1962 were peanut butter, cereals, beans, cashew nuts, almonds, and Brazil nuts.

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[2] This study was a phase of the Southern Regional Research Project, Requirements and Utilization of Selected Nutrients by Preadolescent Children, supported in part by funds appropriated to the U. S. Department of Agriculture under the Research and Marketing Act of 1946, and the Hatch Act, as amended. The Human Nutrition Research Division of the Agricultural Research Service was a cooperator in this project.

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Since no foods of animal origin were included in the 1962 diets, all subjects were fed the same diet (no. 13) containing 39 g of protein from plant sources during the first 6-day period; after this adjustment period, the 4 experimental diets (nos. 9, 10, 11, and 12) were fed.

No supplemental iron was added to the diets in 1956 or 1962, but in 1958 about 33% of the dietary iron was supplied by ferric phosphate incorporated in the bread. An additional 0.3 mg of iron was supplied in a mineral and vitamin supplement. In the last 3 periods of the 1958 study, protein intake was reduced by withdrawing all milk in the diet (120 g/day), and the mineral and vitamin supplement was increased to replace the iron, calcium, phosphorus, thiamine, riboflavin, niacin, and ascorbic acid no longer supplied by the milk. In addition, the daily mineral and vitamin supplement for each girl was diluted with 2.88 g of lactose in the last 3 periods.

Water for drinking and cooking purposes was passed through a zeolite ion exchange unit [5] installed in the water system of the central living quarters, and water used in preparation of sample composites was prepared by passing distilled water through a mixed bed of ion exchange resins.[6]

Samples of food, feces, and vomitus were wet-ashed with sulfuric, nitric, and perchloric acids; and iron content was determined by the o-phenanthroline method of Saywell and Cunningham as modified by Sheets and Ward (6). Differences between duplicate iron determinations in food and feces averaged about 2.5%. Duplicate determinations differing by more than 5% were repeated. Iron absorption in this report indicates the value obtained by substracting the quantity of iron excreted in the feces from the amount of iron consumed. When iron was lost in vomitus, the intake value was reduced by an equivalent amount. Since urinary excretion of iron is normally low, about 1% of the intake (7) and since most available methods yield results of dubious reliability, the iron content of urine was not determined in these studies.

RESULTS

In the 1956 study, the average iron absorption for 12 subjects during 7 alternate 4-day periods ranged from -1.0 to 1.2 mg/day when the diets supplied 6.5 to 7.9 mg of iron and 47 to 88 g of protein daily (table 1). Four of the 12 subjects (one fed diet 2, two fed diet 4, and one fed diet 5) excreted more iron in the feces than was present in the food consumed. Although average iron absorption for each of the other 8 girls was positive, seven of these girls were in negative iron balance in one or more 4-day periods. For all subjects for whom iron excretion exceeded intake by a considerable margin in one or more periods, there were highly significant correlations between iron and nitrogen, and iron and dry matter in the feces (table 2).

In the first 5 periods of the 1958 study, the diet contained about 9.8 mg of iron and 22 g of protein/day. Average iron absorption for all subjects fed this diet was 1.2 mg/day (table 1). Average iron absorption for all subjects increased to 2.1 mg/day during the last 3 periods when milk was replaced with a supplement containing 2.88 g of lactose and amounts of minerals and vitamins approximately equal to those in the milk removed. The difference between the average values for the subjects receiving the 2 diets is statistically significant ($P < 0.01$). Absorption by subject no. 35 was somewhat different from the average as her average iron balance during the first 5 periods was negative, mostly due to a large negative value in period 1. This subject also differed from the other girls in this study in that she required a higher level of dietary protein to maintain a minimal nitrogen retention of 0.3 g/day (3). Because of this higher requirement she was given additional milk in periods 3, 4, 5, 7, and 8 and additional fondant in periods 2 through 8. Her highest iron absorption value occurred in period 6 when her diet contained no milk.

The average iron absorption for every subject in the 1962 study was positive and

[5] High Capacity Cullex Mineral (1.5-cubic foot unit), Culligan, Inc., Northbrook, Illinois.
[6] Amberlite MB-3, Rohm and Haas Company, Washington Square, Philadelphia, Pennsylvania 19105.

TABLE 1

Mean daily protein intake and iron intake, excretion, and absorption by 36 girls 7 to 9 years of age

Subject	Protein intake [1]	Iron			Subject	Protein intake [1]	Iron		
		Intake	Feces	Difference			Intake	Feces	Difference
	g	*mg*	*mg*	*mg*		*g*	*mg*	*mg*	*mg*
				1956 (56 days) [2]					
		Diet 2					Diet 4		
12	47	6.5 ± 1.1 [3]	6.7 ± 0.8	−0.2 ± 0.7	18	59	7.7 ± 0.4	7.5 ± 1.5	0.2 ± 1.5
13	49	7.0 ± 0.4	6.3 ± 0.9	0.7 ± 0.9	19	59	7.7 ± 0.4	8.7 ± 1.8	−1.0 ± 1.9
14	49	7.0 ± 0.4	6.6 ± 0.9	0.4 ± 0.8	20	58	7.3 ± 0.6	8.2 ± 2.8	−0.9 ± 3.3
Means	48	6.8	6.5	0.3	Means	59	7.6	8.1	−0.6
		Diet 3					Diet 5		
15	73	7.2 ± 0.4	6.0 ± 0.5	1.2 ± 0.6	21	88	7.9 ± 0.4	7.1 ± 0.7	0.8 ± 0.9
16	73	7.1 ± 0.4	6.5 ± 0.5	0.6 ± 0.7	22	88	7.9 ± 0.4	8.4 ± 2.8	−0.5 ± 2.8
17	73	7.3 ± 0.2	7.1 ± 0.4	0.2 ± 0.2	23	88	7.9 ± 0.4	7.1 ± 0.8	0.8 ± 1.0
Means	73	7.2	6.5	0.7	Means	88	7.9	7.5	0.4
				1958 (48 days)					
		Diet 8, periods 1–5 (30 days)					Diet 8′, periods 6–8 (18 days)		
24	22	9.8 ± 0.8	8.9 ± 0.3	0.9 ± 0.9	24	18	10.8 ± 0.8	8.9 ± 0.2	1.9 ± 0.9
25	22	9.8 ± 0.8	7.9 ± 0.6	1.9 ± 0.3	25	18	11.0 ± 0.8	8.3 ± 0.2	2.7 ± 1.0
26	21	9.6 ± 1.1	8.2 ± 1.0	1.4 ± 1.0	26	17	10.4 ± 0.8	8.2 ± 0.6	2.2 ± 1.1
27	22	9.8 ± 0.8	8.2 ± 0.9	1.6 ± 1.0	27	18	10.8 ± 0.8	9.8 ± 0.6	1.0 ± 0.4
28	22	9.7 ± 1.1	8.1 ± 0.9	1.6 ± 1.3	28	18	10.7 ± 0.7	8.3 ± 0.5	2.4 ± 1.2
29	22	9.8 ± 0.8	8.3 ± 0.5	1.5 ± 0.9	29	18	10.8 ± 0.8	8.1 ± 0.7	2.7 ± 0.5
30	22	9.8 ± 0.8	8.5 ± 0.5	1.3 ± 0.8	30	18	10.7 ± 0.8	8.9 ± 0.8	1.8 ± 1.4
31	22	9.8 ± 0.8	7.9 ± 0.7	1.9 ± 1.0	31	18	10.8 ± 0.8	8.8 ± 0.5	2.0 ± 1.2
32	22	9.7 ± 0.9	9.3 ± 1.8	0.4 ± 2.4	32	18	10.8 ± 0.8	9.1 ± 0.9	1.7 ± 1.6
33	22	9.8 ± 0.8	8.8 ± 1.1	1.0 ± 1.0	33	18	10.8 ± 0.8	9.6 ± 0.4	1.2 ± 0.8
34	22	9.8 ± 0.8	7.9 ± 1.4	1.9 ± 1.4	34	18	10.8 ± 0.8	8.1 ± 0.9	2.7 ± 1.2
35	24	9.8 ± 0.9	10.5 ± 2.2	−0.7 ± 2.0	35	22	10.9 ± 0.7	8.2 ± 1.6	2.7 ± 2.0
Means	22	9.8	8.5	1.2	Means	18	10.8	8.7	2.1
				1962 (42 days)					
		Diet 10					Diet 12		
46	21	9.8 ± 1.4	9.4 ± 1.2	0.4 ± 0.6	39	37	11.2 ± 0.5	10.7 ± 1.7	0.5 ± 1.5
40	22	10.2 ± 1.0	9.1 ± 1.6	1.1 ± 0.7	45	40	11.9 ± 1.0	11.3 ± 1.5	0.6 ± 1.0
49	22	10.5 ± 0.9	9.7 ± 1.2	0.8 ± 0.5	48	40	11.9 ± 0.9	10.9 ± 1.0	1.0 ± 1.0
Means	22	10.2	9.4	0.8	Means	39	11.7	11.0	0.7
		Diet 9					Diet 11		
42	22	10.3 ± 1.0	9.6 ± 1.0	0.7 ± 1.0	47	40	12.1 ± 1.0	11.6 ± 1.3	0.5 ± 1.0
44	22	10.3 ± 1.0	9.4 ± 0.9	0.9 ± 0.5	43	40	12.4 ± 1.3	11.7 ± 2.3	0.7 ± 0.4
50	22	10.3 ± 1.0	9.7 ± 1.2	0.6 ± 1.3	41	40	12.5 ± 1.2	12.2 ± 1.6	0.3 ± 1.3
Means	22	10.3	9.6	0.7	Means	40	12.3	11.8	0.5

[1] N × 6.25.
[2] The iron content of food and feces was determined in alternate 4-day periods (28 days).
[3] Mean ± SD.

TABLE 2

Correlation of iron with nitrogen and dry matter in feces (1956 study)

Subject no.	Correlations, as r	
	Iron and nitrogen	Iron and dry matter
12	0.73 *	0.21
13	0.46	0.44
14	0.76 **	0.87 **
15	0.36	0.85 **
16	0.25	0.23
17 [1]	0.78	0.44
18	0.92 **	0.90 **
19	0.88 **	0.94 **
20	0.93 **	0.97 **
21	0.69	0.82 **
22	0.92 **	0.98 **
23	0.04	0.28

[1] Data were not available for the first 2 periods.
* Significant ($P \leq 0.05$).
** Highly significant ($P \leq 0.01$).

ranged from 0.3 to 1.1 mg/day when daily intakes of iron were 9.8 to 12.5 mg and of protein, about 22 or 40 g. Period-to-period variation was high, however, and all subjects were in negative balance during one or more periods.

Statistically significant changes in iron absorption were not induced by any of the planned nutrient variables (levels of protein, energy, riboflavin, and niacin) in the 3 studies. The correlation between protein intake and iron absorption expressed as a percentage of intake was not significant for the 3 studies.

Initial hemoglobin values for the 36 subjects in the 3 studies ranged from 11.5 to 15.5 g/100 ml of blood (3, 4). Changes in hemoglobin concentrations during these studies were generally small and were not significantly correlated with iron absorption.

DISCUSSION

With the exception of 4 girls in the 1956 study and one girl during the first 5 periods of the 1958 study who excreted more iron in the feces than was present in the food consumed, 7- to 9-year-old girls in our studies absorbed 2.5 to 25.0% of their dietary iron. These results agree with those reported for healthy children of this age group by other workers (8–11). The high correlations between fecal iron and organic substances in the feces suggest that the high fecal iron values in our 1956 study were not due to extraneous iron contamination of the fecal composites. If the premise is accepted that only a negligible amount of endogenous iron is excreted into the intestinal tract under normal conditions, the amount of iron excreted in the feces would be expected to exceed iron intake only when the subject is in an atypical state or when sampling or analytical errors are involved. Nevertheless, negative apparent iron absorption values are observed frequently in conventional iron balance studies of short duration. Leverton and Marsh (12) reported that 40% of their subjects were in negative iron balance when the daily iron intakes were 5.94 to 7.99 mg. In our 1956 study, 38% of the observations for 4-day periods were negative. Part of the variation was probably due to errors resulting from short, non-consecutive collection periods, infrequent bowel movements by some subjects, and difficulty in separating the carmine-marked and unmarked portions of the feces. Excessive desquamation of cells lining the intestinal tract may have occurred in some subjects who had mild cases of diarrhea in some periods. Sequestration of iron by the epithelial cells of the small intestines for short periods (13) could have contributed also to the period-to-period variation in iron absorption. Since absorbed iron, as estimated by difference, is only a small percentage of dietary iron, small errors in estimations of the amount of iron in food or feces would produce relatively large errors in absorption values. However, if the errors in determination of iron content are random, they should cause occasional over-estimations as well as under-estimations of iron absorption so that averages of absorption over a series of periods should approach true values. Any conventional study of iron absorption, therefore, should be of sufficient duration so that the problems mentioned above will not exert a large influence on the average values.

It is possible that the negative absorption values for two of our subjects (no. 12 and no. 20) were due in part to medication administered for illnesses during the study (3). Subject no. 12 was treated for cold or virus infections during six of the fourteen periods and was treated for poision ivy in 2 additional periods. Subject no. 20 consumed 6.0 mg and eliminated

14.0 mg of iron/day in period 8 during which she complained of an upset stomach and sore throat and was given, for diarrhea, medicine[7] which contained about 15 g of kaolin. The iron content of another batch of this medicine was determined by the same procedure used for assay of the food and fecal samples, and if the iron content of the 2 batches of medicine was the same, this subject received an additional 3 mg of iron/day from the medication. Her average iron absorption would change from − 12.0 to 4.5% of her intake if the value for period 8 were omitted.

Whether the increased iron absorption observed in the 1958 study when the daily protein intake was reduced to 18 g was due to the removal of milk from the diet or to some other factors cannot be determined from these studies; however, other workers (10, 11) have observed a similar influence of milk on iron absorption. Bergeim and Kirch (14) suggested that iron combines with the phosphates and phosphoprotein of milk and does not become soluble until certain degrees of acidity and protein digestion are reached, after which reduction from the ferric to the ferrous forms occurs. If this assumption is true, one would expect milk to exert a marked influence on iron absorption when the diet contained considerable iron in the ferric state as was the case with diet 8. On the other hand, diet 8′ supplied 1.0 mg more of iron/day than diet 8 even though diet 8′ was the same as diet 8, except that the milk had been replaced by a mineral and vitamin supplement containing 0.1 mg of iron. Thus, unplanned differences in composition of the 2 diets, rather than removal of milk, may have been responsible for the difference in iron absorption.

Although the amounts of iron and protein supplied by diet 8 (1958 study) and by diets 9 and 10 (1962 study) were similar, iron absorption was considerably lower when diets 9 and 10, which contained no animal products, were fed. The lower absorption of iron from the all-plant-protein diets may have been due to a lower general digestibility of the diet, a bulkier diet, or to a higher concentration of substances which interfere with iron absorption. It is possible that the iron supplied as ferric phosphate in the bread of the 1958 diets was absorbed more efficiently than was iron from natural foods.

Contrary to observations with laboratory animals (1) our studies failed to demonstrate any consistent effect of level of dietary protein on iron absorption when protein intakes varied from about 30 to 150% of the recommended allowance. It is possible that the influence of dietary protein on iron absorption was masked by other dietary factors or that a critical level of protein intake, beyond which an influence is exerted, was not reached. It is also possible that iron intake was a limiting factor in the 1956 study and any effect of protein intake on iron absorption was obscured in this study.

The Food and Nutrition Board of the National Research Council recently increased the recommended dietary allowance of iron for 6- to 9-year-old children from 10 to 12 mg/day (15). Johnston (16) estimated the daily iron needs of 5-year-old girls to be 0.3 mg for growth, 0.3 mg for maintenance and 0.1 mg for storage; and for 10-year-old girls to be 0.5 mg for growth, 0.4 mg for maintenance and 0.4 mg for storage. On the basis of Johnston's estimations, our results indicate that when physiolgical and other dietary factors are favorable, daily iron intakes of 9 to 12 mg would be sufficient to meet the needs of most 7- to 9-year-old girls, whereas the iron needs of most girls in this age group would not be met by daily intakes of 8 mg or less. Since physiological and dietary factors necessary for good iron absorption are not always favorable, we advocate a dietary allowance of at least 12 mg of iron/day for children in this age group.

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Effect of Reducing Agents on Copper Deficiency in the Chick [1,2]

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ABSTRACT The effect of additions of reducing agents to copper-deficient chick diets was investigated. Ascorbic acid and isoascorbic acid, at 0.1%, and dimercaptopropanol (BAL) at 0.025% of the diet decreased growth and either decreased the elastin content of the aorta or increased mortality in those groups of chicks receiving a copper-deficient, but not a copper-supplemented diet. Diphenylphenylenediamine (DPPD) had no such effect. Ascorbic acid had no effect on zinc or iron deficiency. Dietary ascorbic acid reduced the uptake of Cu^{64} by the liver whether the isotope was given orally or intraperitoneally.

Copper metabolism is influenced by a number of dietary factors other than the level of the element present. It has been found that the addition of small amounts of zinc, cadmium, and silver to a copper-deficient diet resulted in a more severe copper deficiency (1–3). Dick and Bull (4) reported that an increase in dietary molybdenum reduced the copper content of the liver. Later, Dick (5) noted that dietary sulfate was required for molybdenum to exert this influence.

Aside from these influences of known substances, there are evidently other dietary factors which influence copper metabolism. Lewis and Allcroft (6) reported that copper deficiency can occur in lambs and cattle consuming diets apparently normal in copper, molybdenum, and sulfate content. In many instances little correlation has been found between the copper content of the diet and the severity of copper deficiency symptoms when animals are pastured (6).

In an investigation of the influence of dietary factors affecting copper metabolism, attention was directed toward one of the roles associated with copper. In those oxidase enzymes containing copper, such as laccase, ceruloplasmin, and possibly cytochrome oxidase, that part of the metal component which exists in the +2 valence state undergoes reduction and subsequent re-oxidation during the course of the enzymatic reaction (7,8). This dependence upon oscillating valence states for function suggested the possibility that reducing agents might influence copper metabolism. The experiments reported herein were designed to investigate the possibility.

EXPERIMENTAL

The chicks used throughout these studies were White Plymouth Rocks obtained from a commercial hatchery. They were housed in conventional battery brooders with raised wire floors. The watering and feeding troughs were made of stainless steel. The drinking water was demineralized by passing it through an ion exchange resin column. For the zinc-deficient experiment the battery was painted with an epoxy resin paint.

The basal diet used to produce a copper or iron deficiency was based on dried skim milk and glucose as described previously (9). The diet used to produce a zinc deficiency was based on an isolated soybean protein as described by Ziegler et al. (10) except that starch instead of glucose was used.

Copper additions were made by the use of reagent grade $CuSO_4 \cdot 5H_2O$ to supply 25 ppm; iron additions by the use of reagent grade $FeSO_4 \cdot 7H_2O$ to supply 100

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ppm; zinc additions by the use of reagent grade ZnO to supply 50 ppm.

The influence of reducing agents was evaluated by the addition of ascorbic acid, isoascorbic acid, 2-3-dimercapto-1-propanol (BAL), and diphenyl-*p*-phenylenediamine. BAL was added to the diet at 0.025%, the other reducing agents at 0.1%.

In all the experiments, 2 groups of 20 chicks each were fed each diet from the day of hatching.

Elastin determinations were made on the aortas of 3-week-old chicks fed each diet in experiments 1 and 2. The method of elastin measurement and the section of the aorta used were as described previously (9).

Hemoglobin was determined as the oxyhemoglobin.

In the isotope experiment 5 μc of Cu^{64} were given either orally or intraperitoneally. At subsequent times, up to 48 hours after administration of the isotope, 5 chicks fed each diet were killed, the livers removed and weighed, and portions taken for radioactivity determination in a deep-well scintillation counter. The results, corrected for background counts and decay, are expressed as a percentage of the total dose in the whole liver.

Copper determinations on livers from chicks fed a copper-deficient diet and that diet supplemented with ascorbic acid were carried out by the use of an atomic absorption spectrometer.

RESULTS AND DISCUSSION

The results of the studies designed to assess the effects of the reducing agents, ascorbic acid, isoascorbic acid, dimercaptopropanol (BAL), and diphenylenediamine (DPPD), are presented in tables 1 and 2. Elastin determinations were made in experiments 1 and 2 but omitted in 3 and 4 because of the high correlation between mortality and reduced aortic elastin.

The presence of ascorbic acid, isoascorbic acid, or BAL resulted in decreased growth of copper-deficient chicks as well as a reduction in aortic elastin, or an increase in mortality. The ascorbic acids did not depress growth of chicks receiving the copper-supplemented diets. The effect of BAL in inhibiting growth was much less in the presence of adequate copper than in its absence. Neither aortic elastin content nor mortality was affected by these reducing agents in the presence of adequate copper. The presence of these

TABLE 1

Effect of ascorbic acid and DPPD on growth and aortic elastin of copper-deficient chicks

Supplement	3-Week wt [1]	3-Week elastin [2]
	g	*% wet wt of aorta*
Experiment 1		
None	164	8.7
Ascorbic acid, 0.1%	126	6.3
Copper, 25 ppm	242	12.1
Copper, 25 ppm + ascorbic acid, 0.1%	239	12.9
Experiment 2		
None	217	8.5
Ascorbic acid, 0.1%	164	5.5
DPPD, 0.1%	203	8.4
Copper, 25 ppm	246	13.0

[1] Mean of 2 groups of 20 chicks each.
[2] Mean elastin content of 5 chick aortas.

TABLE 2

Effect of ascorbic acid, isoascorbic acid, and BAL on growth and mortality of copper-deficient chicks

Supplement	3-Week wt [1]	3-Week mortality [2]
	g	%
Experiment 3		
None	159	20
Isoascorbic acid, 0.1%	120	35
Ascorbic acid, 0.1%	127	57
Copper, 25 ppm	192	15
Copper, 25 ppm + isoascorbic acid, 0.1%	205	05
Copper, 25 ppm + ascorbic acid, 0.1%	201	05
Experiment 4		
None	182	15
BAL, 0.025%	103	77
Copper, 25 ppm	219	5
Copper, 25 ppm + BAL, 0.025%	187	7.5

[1] Mean of 2 groups of 20 chicks each.

TABLE 3

Effect of ascorbic acid on the copper content of the liver

Supplement	2-Week [1] body wt	Copper content of liver [1]	
	g	*μg/g*	*μg/liver*
None	136	0.74	3.33
Ascorbic acid	110	0.73	2.99

[1] Each value the mean of 20 determinations.

TABLE 4

Effect of ascorbic acid on movement of Cu^{64} into the liver

Hours after isotope administration	Oral		Injected IP	
	Control	Ascorbic acid [1]	Control	Ascorbic acid [1]
	% dose in liver [2,3]	*% dose in liver* [2,3]	*% dose in liver* [2,3]	*% dose in liver* [2,3]
3	4.6	3.0	9.5	10.4
6	6.0	4.0	12.0	13.5
14	10.0	5.2	16.7	11.5
24	7.5	7.5	23.0	13.9
48	10.2	6.8	16.1	10.2

[1] Ascorbic acid at 0.1% in the diet.
[2] Five microcuries given orally and intraperitoneally.
[3] Each value the mean of 5 livers.

3 reducing agents, then, resulted in an increased severity of copper deficiency symptoms.

The presence of DPPD did not result in decreased growth nor decreased aortic elastin content. This reducing agent is relatively insoluble and poorly absorbed. These experiments suggest, therefore, that the effect of reducing agents in increasing the severity of copper deficiency is related to the internal metabolism of the element rather than to an inhibitory effect on absorption. Further support for such a view resulted from the observation on the copper content of the livers of chicks fed the copper-deficient diet alone and this diet supplemented with ascorbic acid (table 3). Although ascorbic acid resulted in decreased growth, the copper content of the liver was not significantly depressed either in concentration or total amount.

To obtain some insight into the possible effect of ascorbic acid on the movement of copper into the liver, radioactive copper was given both orally and intraperitoneally to the control and the ascorbic acid-fed chicks. At various times after the isotope administration the livers of 5 chicks fed each diet were examined for radioactivity. The results are presented in table 4.

The livers from chicks receiving ascorbic acid generally contained less radioactivity than those from the control chicks whether the isotope was given orally or intraperitoneally. This effect was most noticeable after 6 hours. These results suggest that dietary ascorbic acid does influence the movement of copper, either decreasing movement into the liver or increasing movement from the liver. It is possible that the effect of ascorbic acid in increasing the severity of copper deficiency is mediated by this influence on the movement of copper throughout the body.

One other point remained to be examined, the specificity of the reducing agent-copper interaction. It was possible that the effect of these agents was general and not confined to copper. To determine whether this was so, the effect of ascorbic acid on iron and zinc deficiency was examined. The results are presented in table 5. Neither of these deficiencies was enhanced by the presence of dietary ascorbic acid. This observation indicates some degree of specificity for the reducing agent-copper interaction, suggesting that

TABLE 5

Effect of ascorbic acid on zinc and iron deficiencies

Supplement	3-Week wt [1]	Hb [2]
	g	*g/100 cm³*
Experiment 5 (zinc deficiency)		
None	126	
Ascorbic acid, 0.1%	131	
Zinc, 50 ppm	248	
Zinc, 50 ppm + ascorbic acid, 0.1%	243	
Experiment 6 (iron deficiency)		
None	194	4.09
Ascorbic acid, 0.1%	195	4.90
Iron, 100 ppm	238	7.48
Iron, 100 ppm + ascorbic acid, 0.1%	263	7.26

[1] Mean weight of 2 groups of 20 chicks each.
[2] Mean of determinations from 10 chicks.

it is not a generalized phenomenon associated with decreased absorption or transport of elements in general, for instance.

Whether these observations have any relation to manifestations of copper deficiency in animals raised under practical conditions, the results of these studies indicate that the presence of reducing substances in a purified diet can significantly increase the severity of copper deficiency.

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The Utilization of Starch by Larvae of the Flour Beetle, *Tribolium castaneum*

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ABSTRACT The nutritional value of various starches, incorporated in marginal diets, for larval development of the flour beetle, *Tribolium castaneum* (Herbst), was assayed at 32°. Wheat, maize and rice starches were optimal. Larvae died when fed raw potato starch at 32°, but survived and developed slowly at 28°. Development was slightly better with boiled potato starch. The β-amylase limit dextrin "core" of potato starch — containing much esterified phosphorus — was devoid of nutritional value, in contrast with the high nutritional value of β-amylase limit dextrin of commercial amylopectin. The amylolysis of starch by *Tribolium* larvae is attributed mainly to the activity of α-amylase, the characteristics of which were examined. The in vitro hydrolysis of starches is loosely correlated with their nutritional value: amylopectin and β-amylase limit dextrin were well digested, but non-solubilized amylose, only slightly. Boiled potato starch was well digested, whereas raw potato starch was not. The similarity of these in vivo and in vitro results to those obtained with higher animals is noted, and the usefulness of this observation for the bioassay of starches is suggested.

Considerable effort has been directed toward the evaluation of the nutritional value of starch. The importance of assessing this value has increased recently with the development of new starch products for the food industry and the selection of seed varieties containing varying proportions of the different starch fractions. The utilization of starches and starch fractions by rats (1–3) and poultry (4, 5) has been investigated. Some early nutritional experiments were conducted with humans as well (6, 7). Supplementary evidence on the digestibility of starch was obtained by in vitro hydrolysis with various amylases (5, 8–10). Comparable nutritional and enzymic experiments with insects are, however, lacking. Preliminary evidence was presented on the resistance of high-amylose corn to the Angoumois grain moth, *Sitotroga cerealella* (Oliv.) (11), but factors other than the amylose content may have been responsible for this effect, as the experiments were conducted with whole kernels. The purpose of the present investigation was, therefore, to assay the biological value of starches and starch fractions, using as the test organism the Rust-Red flour beetle, *Tribolium castaneum* (Herbst), which is a pest that is most destructive of flours and other stored cereal products. A similarity in response of this organism, to that of higher animals would simplify the biological assay of starch products, whereas dissimilarities would favor the possibility of selecting cereal varieties or developing starch products capable of hindering insect infestation without adversely affecting the nutritional value of such products for humans and livestock.

MATERIALS AND METHODS

Larvae of *Tribolium castaneum* (Herbst) were reared with whole wheat flour in glass jars at the optimal temperature of 32° (12).

In vitro enzymic experiments. Larval enzyme solutions were prepared as described previously (13) at concentrations in accord with preliminary determined, linear amylatic activity. The enzyme solutions were prepared in cold distilled water or buffer and filtered through cotton wool. Enzyme solutions were freshly prepared before each experiment.

Amylatic activity was assayed on soluble starch with the 3,5-dinitro-salicylic acid reagent (14) in a 2-ml reaction mixture. A period of 5 minutes' equilibration at the reaction temperature preceded the 10-minute reaction. Optical density was determined on the boiled and diluted solutions with a Bausch and Lomb Spectronic 20 spectrophotometer at 550 mμ.

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TABLE 1

Composition of basic experimental diets for Tribolium larvae

	%
Carbohydrate	80
Gluten [1]	7
Cellulose [2]	6
Dried yeast [3]	5
Salt mixture no. 2, USP	1
Cholesterol [4]	1

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Hopkin and Williams Ltd., Essex, England.
[4] Reidel-De Haën, AG., Hanover, West Germany.

Growth experiments with Tribolium larvae. The composition of the diets used is shown in table 1. Amounts of 0.25 g diet were assayed in 5 replicates consisting of 10 newly hatched larvae per replicate. After 12 days at 32° the larvae were removed from their diets, and each group was weighed and returned to its container. Age to pupation was determined by daily observations of each container and ET_{50} (days from beginning of experiment to pupation of 50% of the surviving population) was calculated. The results of each series of experiments were analyzed statistically.

RESULTS AND DISCUSSION

Growth experiments with Tribolium larvae. The composition (table 1) and amount of diet (0.25 g/10 larvae) were designed to be marginal, on the assumption that such marginal diets would emphasize slight differences in the nutritional value of the various polysaccharides.[1] *Tribolium* larvae are capable of developing adequately when fed at very low protein levels (fig. 1). A diet of 7% gluten and 6% cellulose was arbitrarily chosen for these experiments. Lowering the carbohydrate level to 60% rice starch and supplementing with an additional 20% cellulose adversely affected larval development, whereas lowering the amount of diet to 0.25 g/10 larvae did not (table 2).

Basic experimental diets were compounded with various proportions of amylopectin and amylose as the source of carbohydrate. Amylopectin is far superior to amylose in fulfilling the carbohydrate requirements of the developing larvae, al-

[1] This assumption was verified in several growth experiments conducted at 28° with 1 g diet/10 larvae and a 13% gluten level. Thus, for example, the average weight of larvae under such experimental conditions, with glycogen or dextrin assayed at an 80% level, was 1.97 and 1.93 mg, respectively, as compared with 1.17 and 1.50 mg weight with marginal diets (table 3).

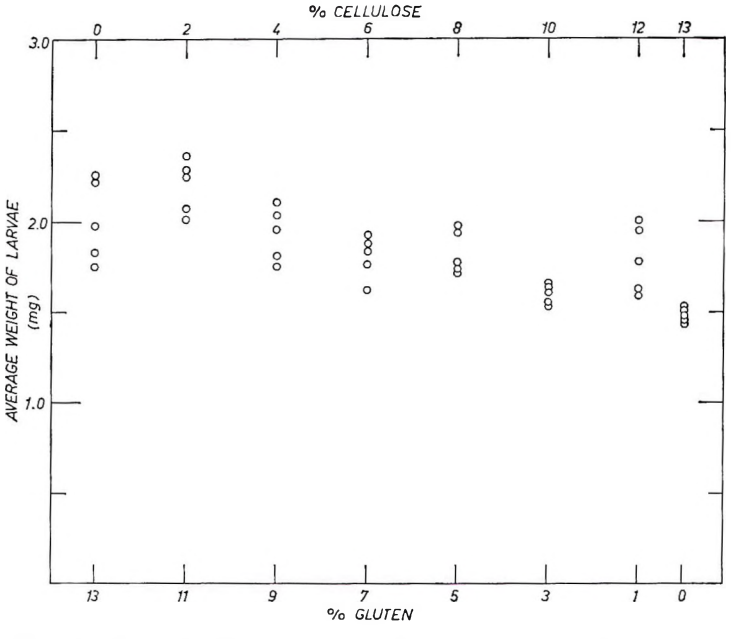

Fig. 1 Effect of protein level on larval development of *Tribolium*.

though the amylose is not completely devoid of nutritional value. This conclusion is reached by comparing the results with amylopectin:amylose diets to those with amylopectin:cellulose diets (fig. 2).

To verify the results obtained with high-amylose diets, 2 other amylose preparations (designated amylose I and II and differently prepared) were compared with the commercial amylose preparation (table 3). Although large differences were evident in the response of *Tribolium* larvae to the 3 different amylose preparations, in all cases development was significantly less than with the amylopectin control diet.

Comparable results have been reported on the utilization of starch fractions by the rat. The starch of high-amylose corn was digested to the extent of 66 to 77%,

TABLE 2

Effect of lowering starch level or decreasing the amount of food on larval development of Tribolium

Diet	Amount/ 10 larvae	Avg wt of larvae	Larval survival	ET_{50}[1]
	g	*mg*	*%*	*days*
7% Gluten + 80% rice starch [2]	1.0	1.93 ± 0.096 [3,4]	82	16
7% Gluten + 60% rice starch [5]	1.0	1.57 ± 0.219 [3]	84	16
5% Gluten + 80% rice starch	0.5	2.17 ± 0.125	96	15
5% Gluten + 80% rice starch	0.25	2.02 ± 0.098	96	16.5
5% Gluten + 80% rice starch	0.1	1.48 ± 0.113 [3]	88	— [6]

[1] Days from beginning of experiment to pupation of 50% of the surviving population.
[2] The British Drug Houses Ltd., Poole, England.
[3] $P < 5\%$.
[4] ± SE.
[5] Supplemented with 20% cellulose.
[6] None of the surviving larvae pupated.

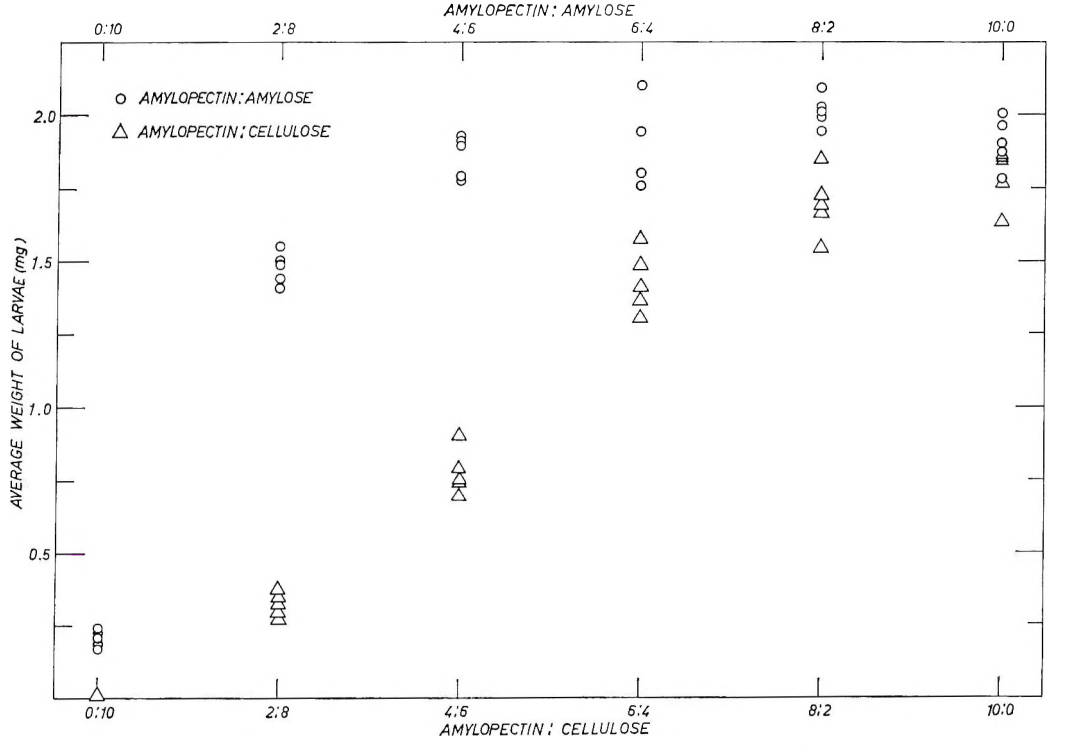

Fig. 2 Effect of amylopectin and amylose on larval development of *Tribolium*.

TABLE 3

Effect of various polysaccharides and starch fractions on larval development of Tribolium

Polysaccharide assayed	Avg wt of larvae	Larval survival	ET_{50}[1]
	mg	%	*days*
Commercial amylose [2]	0.22 ± 0.030 [3]	84	— [4,5]
Amylose I	1.32 ± 0.098	88	22 (4 replicates) [5]
Amylose II	0.15 ± 0.029	66	30 (3 replicates) [5]
Amylopectin control [2]	1.98 ± 0.134	100	19
Potato starch (raw) [6]	0	0	—
Potato starch (boiled) [7]	1.07 ± 0.087 [8]	75	21
Potato starch β-amylase limit dextrin [9]	0.01 [8]	18	—
Glycogen [10]	1.17 ± 0.108 [8]	96	20
Dextrin [6]	1.50 ± 0.090 [8]	96	20
Rice starch [11]	1.89 ± 0.105	98	17
Maize starch [11]	1.90 ± 0.125	98	17
Wheat starch [12]	1.98 ± 0.051	96	17
Inulin [11]	0	0	—
β-Amylase limit dextrin [13]	1.85 ± 0.076	96	16

[1] Days from beginning of experiment to pupation of 50% of the surviving population.
[2] California Corporation for Biochemical Research, Los Angeles.
[3] ± SE.
[4] None of the surviving larvae pupated.
[5] $P < 5\%$.
[6] Allied Chemical and Dye Corporation, New York.
[7] Prepared by boiling potato starch in water and lyophilizing the solution.
[8] $P < 1\%$.
[9] Prepared from a boiled 4% solution of potato starch in 0.01 M phosphate buffer, pH 6.2. Beta amylase (barley, standardized at 2,000°, Lintner-Mann Research Laboratories, Inc., New York) was added in 3 batches of 50 mg/8 liters solution, initially, after 5 hours and after 48 hours. The reaction continued for 48 hours at 39° under constant stirring, an additional 24 hours while dialyzing against the above buffer and a final 24 hours while dialyzing against distilled water. Toluene was present throughout this whole period as a precautionary measure. The digest was boiled in order to inactivate the β-amylase, and lyophilized.
[10] Nutritional Biochemicals Corporation, Cleveland.
[11] The British Drug Houses, Essex, England.
[12] Prepared according to Whelan (25).
[13] Prepared from amylopectin according to Applebaum (21).

whereas that of normal corn was digested to the extent of 95% (2). Similarly, the amylopectin fraction of potato starch was better utilized by the rat than the amylose fraction (3).

The nutritional value of various carbohydrates for larval development of *Tribolium* is summarized in table 3. Raw potato starch did not support larval development at all when experiments were conducted at 32° (table 3). However, in comparable experiments conducted at 28°, larval survival, after 12 days of development, was 98% and the average larval weight was 0.36 mg. Presumably, raw potato starch is slightly available for larvae developing at 28° but not sufficiently so to supply the energy required for the higher level of metabolism of larvae growing at 32°. The indigestibility of raw potato starch is probably due, to a large degree, to the resistance of the intact starch granule, since boiled potato starch did support larval development, although less than other starches. The poor availability of potato starch for poultry (4) and rats (1) has been reported. The esterified phosphorus present in potato starch might be responsible for the lower availability of boiled potato starch. The β-amylase limit dextrin of potato starch contains all this phosphorus (15) and this fraction barely supported larval development. This is in contrast with the optimal development on β-amylase limit dextrin of commercial amylopectin. The absence of development with inulin is in accord with the reported absence of inulinase in *Tribolium* larvae (16).

Optimal conditions for larval amylatic activity in vitro. The optimal substrate concentration was determined on soluble starch in the range of 0.05 to 4% concentration in the reaction mixture. A concentration of 2% starch was satisfactory for maximal amylatic activity.

Composite amylatic activity was determined on 2% soluble starch in 0.01 M

acetate buffer in the pH range of 3.7 to 5.2 and in 0.01 M phosphate buffer in the pH range of 5.7 to 7.4. This range was subsequently examined in the presence of 5×10^{-4} M $CaCl_2$, as the activator of amylatic activity. The $CaCl_2$-activated amylase exhibited a broad optimum in the range of pH 4.6 to 5.2, whereas non-activated amylase exhibited a more narrow optimal peak at pH 5.2 (fig. 3).

To find out the degree of glycosidase activity in the enzyme preparations, determinations were made under identical conditions in the range of pH 3.7 to 5.7, with 2% sucrose as the substrate. Glycosidase activity was relatively low and exhibited no optimal peak in the optimal pH range for amylase activity (fig. 3). The pH optima of different insect amylases range between 5.5 and 9.5 (17).

Optimal temperature during a reaction period of 10 minutes was determined in the range of 5 to 55° at pH 5.0. The optimal temperature under these conditions is 35°, which is the optimal temperature for larval development as well (12). In comparison, the optimal temperature for *Tenebrio* amylase is 25° (18), which in the case of *Tenebrio* is optimal for development also.

The effect of various activators and inhibitors on *Tribolium* amylatic activity is summarized in table 4. The results most conclusive in characterizing the *Tribolium* amylase as an α-amylase is the inhibition by the α-amylase inhibitor from wheat, as this inhibitor has no effect on β-amylase (19). The activation of *Tribolium* amylase by $CaCl_2$ strengthens this conclusion, for such activation is encountered in many α-amylases (20) including *Tenebrio* amylase (21).

Sulfhydryl compounds are recognized as activators of plant β-amylases (22, 23). As glutathione and mercaptoethanol slightly inhibit *Tribolium* amylase SH-activation is certainly not essential. The inhibitory effect of ethylenediamine tetraacetate (EDTA) may be attributed to chelation of Ca^{++} from the amylase molecule. Heavy metals such as Hg^{++} are recognized as non-specific inhibitors of enzymic activity. The high degree of inhibition encountered in this case would be expected. Ascorbic acid is known to inhibit plant β-amylase (24). The slight inhibition encountered here cannot, however, be attributed to wheat β-amylase originating from the diet of the insects, as neither *p*-hydroxymercuribenzoate nor ascorbic acid were found to

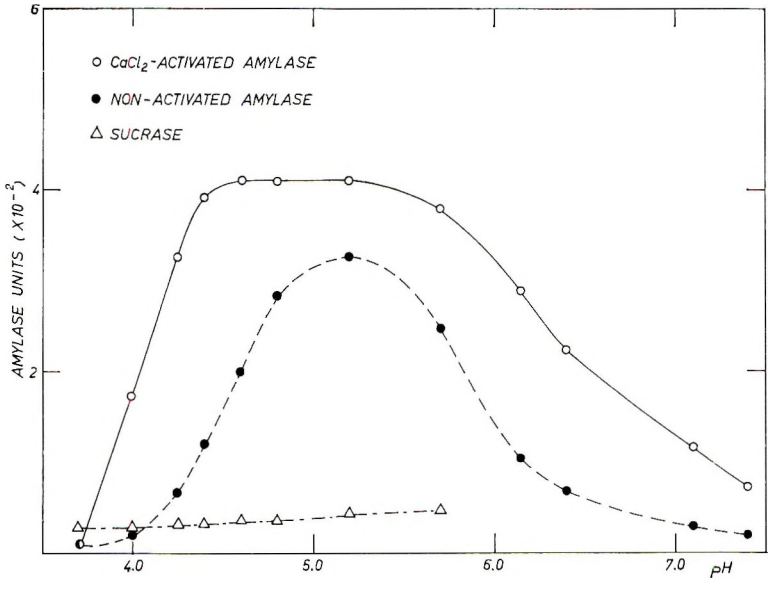

Fig. 3 The pH dependence of *Tribolium* amylatic activity.

induce further inhibition of activity in the presence of α-amylase inhibitor.

Incubation at 35° of enzyme solutions alone, for periods up to 4 hours prior to the assay of amylase activity, resulted in an approximately linear decrease of activity. Incorporation of 2×10^{-3} M $CaCl_2$ was not effective in stabilizing this activity.

No loss of amylase activity was noted after dialysis in the cold against either 2×10^{-3} M $CaCl_2$ or distilled water.

In the initial period of digestion of various starch fractions and glycogen by *Tribolium* amylase (fig. 4), solubilized amylose, amylopectin and the β-amylase limit dextrin of amylopectin were hydrolyzed at

TABLE 4

Effect of various compounds on composite amylatic activity of Tribolium larvae

Compound added	Conc in reaction mixture	Amylase units [1]	Inhibitor units [2]
$CaCl_2$ [3]	5×10^{-4} M	6.8×10^{-2} [4]	
Glutathione [5]	4×10^{-4} M		3.4×10^{-3}
Ascorbic acid [6]	4×10^{-4} M		3.5×10^{-3}
EDTA [7]	2×10^{-3} M		4.4×10^{-3}
2-Mercaptoethanol [8]	6.25×10^{-5} M		5.2×10^{-3}
$HgCl_2$ [9]	1.25×10^{-3} M		4.8×10^{-2}
α-Amylase inhibitor [10]	0.25% (w/v)		4.5×10^{-2}
Initial amylatic activity		4.8×10^{-2}	

[1] Amylase unit = increase of 1 unit of optical density at 550 mμ per 1-minute digestion under optimal experimental conditions.
[2] Inhibitor unit = amylase unit inhibited.
[3] Allied Chemical and Dye Corp., New York.
[4] Or an addition of 2×10^{-2} amylase units to the initial amylatic activity in the absence of activation or inhibition.
[5] Nutritional Biochemicals Corporation, Cleveland.
[6] Fluka AG., Switzerland.
[7] Disodium ethylenediamine tetraacetate, Fisher Scientific Company, Fair Lawn, New Jersey.
[8] Matheson Coleman and Bell, Cincinnati, Ohio.
[9] May and Baker Ltd., Dragenham, England.
[10] α-Amylase inhibitor was prepared by extracting one part of whole-wheat flour with 100 parts of distilled water at room temperature, with constant stirring. The mixture was then centrifuged at about 3000 rpm for 15 minutes and the supernatant collected and brought to a boil quickly to inactivate wheat β-amylase. The boiled solutions were cleared by filtering through Whatman no. 2 filter paper.

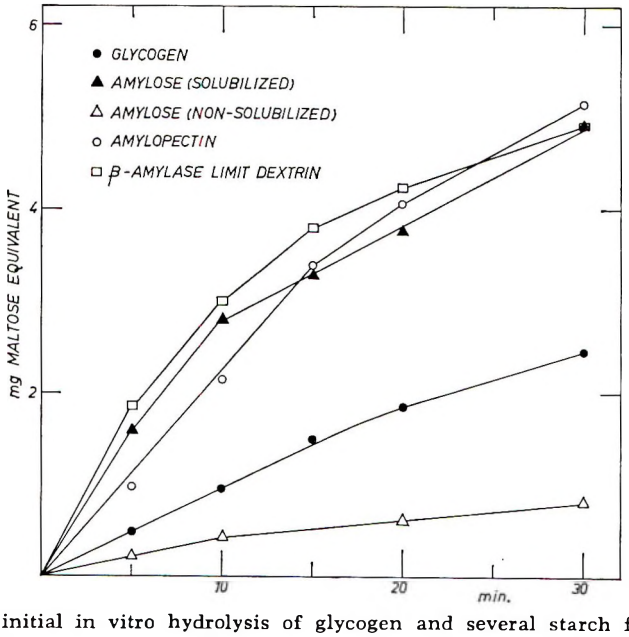

Fig. 4 The initial in vitro hydrolysis of glycogen and several starch fractions by *Tribolium* amylatic enzymes.

TABLE 5

Degree of hydrolysis of various substrates by Tribolium larval amylase [1]

Substrate	Hydrolysis
	%
Amylose (solubilized) [2,3]	64
Amylose (non-solubilized) [2,4]	18
Amylopectin [2,5]	85
Dextrin [5,6]	50
Glycogen [5,7]	57
β-Amylase limit dextrin [5,8]	79
Raw potato starch [4,6]	0
Boiled potato starch [6]	86
Potato starch β-amylase limit dextrin [9]	64

[1] All reaction mixtures and controls were buffered to 0.01 M acetate buffer pH 5.1 and 5×10^{-4} M $CaCl_2$ and incubated at 35° for 3 hours. The concentration of substrate in the reaction mixture was 0.5%.

[2] California Corporation for Biochemical Research, Los Angeles.

[3] Solubilized by wetting 1 g with 2 ml of absolute ethanol, adding 4 ml of 10% NaOH and heating in a boiling water bath. The solution was then neutralized with 6 N HCl and the volume brought to 100 ml.

[4] Assayed by distributing 10 mg into each reaction mixture replicate, and conducting the incubation under constant shaking.

[5] Dissolved in water at room temperature.

[6] Allied Chemical and Dye Corporation, New York.

[7] Nutritional Biochemicals Corporation, Cleveland.

[8] Prepared from amylopectin as described by Applebaum (21).

[9] Prepared from potato starch as described in table 4.

the same rate. Glycogen was hydrolyzed significantly less, a result similar to that observed for *Tenebrio* amylase (21), and non-solubilized amylose was barely hydrolyzed. The solubilized amylose subsequently retrograded from solution and this may explain the lower level of final digestion obtained after 3-hour incubation (table 5). The complete absence of hydrolysis of raw potato starch and the high degree of hydrolysis of the boiled potato starch solution (table 5) is in accord with the reported action of amylases from other sources (5, 8, 10). The raw potato starch suspension and boiled potato starch solution were microscopically examined prior to incubation with the larval enzyme solution. In the former, typical intact starch granules were present, whereas in the latter these granules were disrupted. The high digestibility of β-amylase limit dextrin of amylopectin again confirms the α-amylase nature of *Tribolium* amylase.

The use of *Tribolium* in the biological assay of starches and industrial starch products is suggested because of its developmental response to polysaccharides, which is similar to that of higher animals. Growth experiments with this beetle are simple to perform, and significant results can be obtained within a short period.

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Effect of Dietary Vitamin D and Protein on Free Amino Acids and Lipids in Selected Rat Tissues [1]

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ABSTRACT Weanling male rats were fed for 6 weeks vitamin D-deficient rations which contained 2 levels of protein with varying levels of supplemental calciferol. Vitamin D decreased the concentration of free lysine but had no apparent effect on free methionine or valine in plasma. Increasing the level of the vitamin significantly increased the concentration of cholesterol and total lipids in liver of rats fed 9% of casein but had no significant effect on that of animals fed 18% of casein. Increasing the protein intake decreased the level of lipids in liver of animals fed no vitamin D supplement or either level of the supplementary vitamin. The results suggested that amino acid-calciferol ratio may be important in lipid metabolism.

The work of Colovos et al. (1) showed that insufficient vitamin D in the ration of calves significantly decreased protein utilization and inhibited the growth rate. Since then, Jonxis and Huisman (2) observed that vitamin D_3 therapy reduced the amount of amino acids excreted by rachitic children. Steenbock and Herting (3) reported that vitamin D may be concerned with metabolism of soft tissue. Other workers have observed that addition of calciferol to the diet of experimental animals increases the concentration of cholesterol in body tissues (4, 5). On the basis of these observations, the present study was made to determine the effect of dietary vitamin D on the concentration of certain free amino acids in plasma and cholesterol and total lipids in liver tissue of rats fed at 2 levels of protein.

EXPERIMENTAL

Male rats weighing 50 to 70 g of the Sprague-Dawley strain, were housed in individual galvanized metal cages with wire bottoms and given food and water ad libitum during the 6-week experimental period. Three groups of 12 rats each, similar in weight and littermate distribution each received diets which contained 9% of protein and zero, 100 or 10,000 IU of vitamin D (calciferol) per week. Three more groups received 18% of protein with corresponding amounts of vitamin D. The vitamin D supplements, which were administered orally 3 times/week, were prepared by the addition of weighed quantities of calciferol to weighed amounts of cottonseed oil to give the desired concentrations of calciferol and to provide a comparable amount of carrier for each group of animals. The complete diets are shown in table 1.

Blood from non-fasted, decapitated rats was collected in 15-ml centrifuge tubes which contained 3 mg of sodium heparinate (6). Equal volumes of plasma from 4 rats in each dietary group were pooled. Protein-free filtrates were prepared (7) and assayed microbiologically for lysine, methionine and valine (8).

Liver tissue from each rat was analyzed for cholesterol (9) and total lipids of liver were measured by the method of Bloor (10) as modified by Okey and Lyman (11).

RESULTS

Free amino acids in plasma. The plasma levels of lysine, methionine, and valine of the animals receiving the different diets are shown in table 2. Supplemental feeding of 100 or 10,000 IU of vitamin D/week decreased the concentration of plasma lysine at both levels of protein intake, but had no significant effect on methionine or valine (F value for methionine = 0.88, for valine = 0.66, required

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TABLE 1

Composition of diets

	9% Protein	18% Protein
	%	%
Casein [1]	9	18
Sucrose	78	69
Cottonseed oil [2]	5	5
Salt mixture [3]	4	4
Cellulose [4]	4	4
		mg/kg
Thiamine·HCl		5.0
Pyridoxine·HCl		2.5
Riboflavin		8.0
Ca pantothenate		20.0
Niacin		10.0
Biotin		0.1
Folacin		2.0
Vitamin B_{12}		0.02
Inositol		100.0
p-Aminobenzoic acid		100.0
Menadione		0.5
Choline chloride		1300.0
Vitamins A, E [5]		

[1] Vitamin-Free Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Wesson Oil, Wesson Sales Company, Fullerton, California.
[3] U. S. Pharmacopoeia, ed. 14, p. 789, 1950.
[4] Solka Floc, Brown Company, San Francisco.
[5] Cottonseed oil-vitamin D preparation provided 1000 IU vitamin A acetate and 0.8 mg α-tocopherol/rat/week.

for $P < 0.05 = 3.88$). The effect of vitamin D on plasma lysine was significant at the 1% level. Increasing the casein level of the diet significantly increased ($P < 0.01$) the concentration of plasma methionine and valine but had no significant effect on plasma lysine ($F = 1.98$, required for $P < 0.05 = 4.75$).

Liver cholesterol and total lipids. Increasing the dietary protein from 9 to 18% significantly decreased ($P < 0.01$) the concentration of liver cholesterol and of total lipids (table 3). When 9% of casein was fed, the concentrations of liver cholesterol and of total lipids increased when vitamin D was added to the diet. The effect was highly significant ($P < 0.01$). Vitamin D had no effect on the concentration of lipids in liver of animals fed 18% of protein.

Growth. A summary of the growth data is shown in table 3. At the close of the experimental period the total weight gain of animals fed 9% of casein was not significantly affected by the level of dietary vitamin D. However, the mean body weight of animals fed no vitamin D supplement had been less than that of the other 2 groups until the last week of the

TABLE 2

Effect of vitamin D on free amino acids in plasma of rats fed at 2 levels of protein

Dietary protein level	Vitamin D (calciferol)	Free amino acids [1]		
		Lysine	Methionine	Valine
% of diet	*IU*	*μg/ml*	*μg/ml*	*μg/ml*
9	0	70.7 ± 0.4	7.9 ± 0.4	20.8 ± 2.1
9	100	66.6 ± 1.4	6.1 ± 0.1	21.5 ± 1.5
9	10,000	66.5 ± 1.4	6.8 ± 0.1	24.3 ± 3.4
18	0	77.0 ± 1.9	11.8 ± 0.4	33.7 ± 2.6
18	100	63.9 ± 3.9	12.4 ± 0.3	29.7 ± 3.6
18	10,000	70.5 ± 2.1	12.2 ± 0.5	33.1 ± 2.8

[1] Mean value of 3 pooled samples ± SE of mean.

TABLE 3

Influence of dietary vitamin D on weight gain, cholesterol and total lipids in liver of rats fed at 2 levels of protein

Dietary protein level	Vitamin D (calciferol)	Body wt gain [1]	Liver wt [1]	Total cholesterol [1]	Total lipids [1]
% of diet	*IU*	*g*	*g*	*mg/g*	*mg/g*
9	0	100 ± 3	6.5 ± 0.40	2.34 ± 0.2	43.2 ± 2.5
9	100	98 ± 4	6.8 ± 0.14	3.61 ± 0.3	60.5 ± 5.2
9	10,000	96 ± 4	7.1 ± 0.08	5.32 ± 0.7	81.6 ± 9.9
18	0	214 ± 8	11.0 ± 0.05	1.86 ± 0.04	36.6 ± 0.4
18	100	257 ± 5	13.3 ± 0.05	1.90 ± 0.05	36.1 ± 0.5
18	10,000	264 ± 5	13.5 ± 0.08	1.95 ± 0.04	37.9 ± 0.6

[1] Mean of 12 animals ± SE of mean.

experiment when it surpassed them. When 18% of casein was fed, the weight gain of animals fed 100 IU of the vitamin was significantly greater ($P < 0.01$) than that of animals receiving no vitamin supplement. The weight gain of animals fed 10,000 IU of the vitamin did not differ significantly from that of animals receiving the lower level of vitamin supplement. The weight gain of animals fed 18% of casein was more than twofold that of corresponding animals fed 9% of casein.

During the last 2 weeks of the experiment a marked change occurred in the appearance of the animals fed 9% of casein plus calciferol. The rats showed alopecia over parts of the body and the remaining hair was matted and coarse. The irregular appearance of the fur was most pronounced in 6 animals fed 100 IU of vitamin D and 4 animals fed 10,000 IU of the vitamin. The fur of all animals fed 9% of casein with no vitamin D supplement was smooth. At the end of the experimental period, the animals fed 18% of protein were all healthy in appearance.

DISCUSSION

Since previous work (12) indicated that the level of free lysine, methionine and valine in plasma of the rat may be affected by certain dietary nutrients, these amino acids were selected for the study of the relationship between dietary vitamin D and free amino acids in plasma. The results suggest that the elevated level of lipids in liver of animals fed 9% of protein compared with those fed 18% of casein were due, in part, to the decreased levels of valine and methionine in plasma. The effect of dietary methionine on lipid deposition in tissues has been well documented (12–15). Sidransky and Baba (16) reported that rats force-fed a diet deficient in valine developed fatty livers. The data for animals fed 9% of protein suggest further that the change in ratio of plasma lysine to other amino acids which resulted from the addition of vitamin D to the diet may have been the critical factor which precipitated the higher levels of liver lipids in the animals fed this supplement. Reports from other laboratories have shown a relationship between dietary lysine and the concentration of lipids in tissues (16–18). In the present study the decreased level of lysine in plasma of animals fed 18% of protein plus vitamin D had no effect on lipid tissues. This suggests that amino acids or other nutrients not included in the analyses may also be related to vitamin D and lipid metabolism at low levels of protein intake. In a recent evaluation of mineral mixtures used for experimental studies Williams and Briggs (19) pointed out that the USP XIV salt mixture used in this study is deficient in zinc and supplies less than the National Research Council (20) recommendation for certain other minerals. The apparent healthy condition of all animals fed 18% of protein indicated that zinc per se was not a contributing factor in observations of this study.

Whether the use of a higher level or a different type of fat in the present study would have shown a relationship between vitamin D and lipids in animals fed 18% of protein is unknown. Nath and Harper (5) obtained evidence that the addition of 1 mg of calciferol to 100 g of a diet which contained 25% of hydrogenated coconut oil and 20% of casein with added methionine increased deposition of cholesterol and total lipids in liver of rats. In the studies of Lee and Herrmann (4) the addition of vitamin D to a diet which contained 66% of sucrose, 18% of casein and 8% of hydrogenated oil increased serum and liver cholesterol of normal rats. With a diet of commercial laboratory chow [2] and added corn oil these workers showed that vitamin D had no effect on lipid concentration in tissues of the rat.

The use of pooled samples was necessary because of small amounts of blood but the data presented indicate that dietary vitamin D may be related to lipid and amino acid metabolism.

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Excretion of Thiamine and its Metabolites in the Urine of Young Adult Males Receiving Restricted Intakes of the Vitamin [1]

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ABSTRACT Eight young men consuming a 2800-kcal diet consisting of 80 g protein, 100 g fat and 400 g carbohydrate and providing 0.11 to 0.18 mg thiamine /day, developed clinical symptoms of thiamine deficiency in 9 to 27 days. Thiamine excreted in the urine decreased to less than 50 μg/day at the sixth day and to undetectable levels by the eighteenth day of depletion. Low-level repletion (0.54 to 0.61 μg/day) for 12 days failed to produce detectable levels of thiamine in the urine. Red blood cell transketolase activity declined with progressive thiamine deficiency and returned to normal levels with low-level repletion. The excretion of the pyrimidine and thiazole moieties of thiamine as metabolites of the vitamin appeared to increase above the levels found with the use of a diet with sufficient thiamine (about 2 mg/day), and was reduced to control levels following low-level repletion. The pyrimidine moiety in the deficient individual appears to be further catabolized. There appears to be a body store of thiamine which is utilized during a period of deficient intake. Serum cholesterol, nonesterified fatty acids, blood phospholipids, creatinine and hematocrit did not change appreciably during thiamine deficiency. The biochemical pattern for thiamine deficiency in human adults is described.

In seeking biochemical indices of nutritional status, many investigators have measured the relationship between the amounts of a vitamin ingested and the amounts of the vitamin excreted in the urine. Thiamine balance studies using normal and deficient subjects have demonstrated that: 1) as the deficiency progresses, the amount of thiamine excreted in the urine diminishes, and 2) there is a direct correlation between the extent of deficiency and the retention of a repletion dose of thiamine. Thus, by the use of these criteria, it was thought possible to establish the level of nutritional status in terms of thiamine status. It was early recognized that the amount of thiamine excreted in the urine might reflect only recent intakes of the vitamin rather than the degree to which the subject's tissues contained adequate levels of thiamine. The excretion values were therefore considered unreliable as indicators of thiamine levels in the body. With respect to the retention of thiamine in deficient individuals, it has been difficult to establish numerical excretion values above which the subject was deemed normal, since this required an accurate assessment of the degree of deficiency.

Recently (1), it has been shown that an enzyme found in the red blood cell requires thiamine pyrophosphate for its normal functioning in the pentose shunt pathway. This enzyme, transketolase, has been found to be sensitive to the levels of thiamine in the body and has therefore been used as an index of thiamine sufficiency. Furthermore, recent work (2) on a method to measure the amount of thiamine and its metabolites in urine was reported, thus making possible the measurement of thiamine in the urine as well as the thiamine that was split either by metabolic processes or by virtue of thiamine's instability in the body's media. The work being reported here was designed to in-

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[1] Unless otherwise specified, the word "metabolites" referred to in this paper signifies those compounds excreted in the urine which, when incubated with dried active baker's yeast (2), form a compound which behaves like thiamine in the USP or Association of Official Agricultural Chemists' fluorometric assay for thiamine.

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vestigate the effect of thiamine deficiency on the red blood cell transketolase, the excretion pattern of thiamine and its metabolites in urine, as well as the measurement of other biochemical parameters which have not yet been implicated in this deficiency syndrome.

METHODS AND EXPERIMENTAL PLAN

Subjects. Eight normal young men serving as volunteer test subjects in a metabolic ward were fed diets containing known amounts of thiamine with controlled caloric intake.

Diet. The diet consisted of 80 g protein, 100 g fat and 400 g carbohydrate, providing a daily intake of 2800 kcal. The diets were essentially the same as those of Anderson et al. (3) except that substitutions were made with foods which were available and indicated by analysis that they did not increase the amount of thiamine in the diet. The diets were made up as menus 1, 2 and 3; each menu provided 3 meals per day plus a snack before going to bed. The menus were rotated so that the same foods were offered every 3 days. As the deficiency progressed, nausea and emesis occurred so that the subjects did not eat all of the food offered them, or lost some of that eaten. Analyses of the menus were performed on composites that contained representative portions and were homogenized before assay sampling. The thiamine content of the menus was as follows: menu 1, 0.11 mg; menu 2, 0.16 mg; menu 3, 0.18 mg. Water was permitted ad libitum.

Activity. The activity of the subjects was essentially ad libitum with attempts to minimize variations from man to man and day to day.

Medications. The following medications were prescribed as needed: for headache, aspirin or aspirin-phenacetin-caffeine; for nausea, anti-spasmodic mixture consisting of tincture of belladonna, 8 to 10 drops and 10 mg of phenobarbital; for emesis, chlorpromazine, 25-mg tablets, 1 to 3/dose.

Physical examinations. A weekly physical examination was performed on each subject with special reference to a check list of signs for thiamine deficiency. An electrocardiogram was taken weekly during the depletion period. Each subject was urged to keep a diary of any symptoms noted.

Experimental design. The 51-day experiment was divided into one 3-day and eight 6-day balance periods with periods 1 (3 days) and 2 being used for control, periods 3 through 6 for depletion and periods 7 through 9 for repletion. During the control period, each man received 1.6 mg of thiamine hydrochloride in addition to the thiamine in his daily diet; during the period of repletion, the subjects were given supplements of 0.43 mg of thiamine/day so that their intake, including that in the diet, was 0.54 to 0.61 mg/day, a level considered just above the deficient intake (4). During the entire study the subjects were given daily supplements as follows: riboflavin, 2 mg; nicotinamide, 20 mg; pyridoxine, 2 mg; calcium pantothenate, 10 mg; folic acid, 150 μg; vitamin B_{12}, 1 μg; ascorbic acid, 30 mg; choline, 0.5 g; biotin, 10 μg; vitamin A, 5,000 IU; vitamin D, 1,000 IU; α-tocopherol, 20 mg; menadione, 0.5 mg; calcium gluconate, 0.84 g.

Twenty-four-hour urine samples were collected on the sixth day of each period. The urine collection bottles contained 5 ml of concentrated sulfuric acid and were stored in the refrigerator during the 24-hour period. At the end of the collection, the pH was adjusted to 1 to 2 with 1:1 H_2SO_4 using thymol blue as an external indicator. The final volume was recorded, and an aliquot drawn off and stored in the freezer until analysis was performed.

Methods. The urine samples were assayed for thiamine and metabolites using the method previously described (2). Blood for the transketolase determinations was drawn into stoppered heparinized tubes and analyzed by the method of Wolfe et al. (1) as modified by Sauberlich and Bunce (5), measuring the loss of ribose-5-phosphate as an index of enzyme activity. Nonesterified fatty acids were determined by the method of Dole (6), serum cholesterol by the method of Searcy and Bergquist (7), lipid phosphorus by the method of Fiske and Subbarow (8) and urine creatinine by the method of Bonsnes and Taussky (9).

RESULTS

General comments. The subjects did not enjoy consuming the sweet, monotonous, high carbohydate diet, but were asymptomatic until after thiamine was withdrawn on the ninth day of the study. During the next 2 weeks, subjective symptoms appeared with increasing frequency in 5 of 8 subjects. They consisted of general malaise, headache, nausea, constipation and generalized aching of the muscles. Vomiting was common during the third week of depletion. Three subjects remained completely asymptomatic throughout the study despite biochemical evidence of deficiency.

Physical observations. Weekly physical examinations were carried out during the entire study. The earliest abnormality appeared within 9 days of depletion and consisted of a sinus tachycardia at rest. This symptom was prominent in 3 of 8 subjects and persisted until thiamine repletion was begun. Muscle strength diminished in the legs of 2 subjects and was elicited by having the individual perform full knee bends. Changes in deep tendon reflexes were noted in 4 of 8 subjects. This change consisted of diminished activity of the ankle jerk (2 subjects), knee jerk (3 subjects), biceps and triceps reflex (4 subjects) and became apparent after 3 weeks of depletion. Sensory abnormality was a rare observation, but consisted of a spotty diminution of pinprick sensation over the extremities in 3 of 8 subjects. This questionable abnormality appeared during the third week of thiamine deprivation. Within one week of thiamine repletion, there was a disappearance of all abnormal physical symptoms. Symptoms of general malaise and others previously noted persisted for 2 weeks, then gradually cleared.

Biochemical observations. The urine volume (fig. 1(A)) of the subjects did not differ significantly during the study when the control, depletion and repletion outputs were compared.

The metabolites excreted (fig. 1(B)) increased during the thiamine depletion, although not significantly so when measured by the paired *t* test, and remained elevated through the early phases of low-level repletion and returned to control values on the last day of the study. By the sixth day of depletion the excretion of thiamine was reduced to very low levels and became undetectable by the eighteenth day (table 1). This changed on the first and second day of repletion with the excretion of barely detectable amounts. From the third to the ninth day of repletion there was, again, no detectable thiamine in the urine. From the ninth through the twelfth day of repletion, there was evidence of the excretion of small amounts of thiamine. During the depletion period, and for most of the repletion period, the thiamine metabolites continued to be excreted at elevated levels when compared with excretion of the controls.

The transketolase values (fig. 1(C)) showed a significant decrease as the deficiency progressed. By days 27 and 33

TABLE 1

Thiamine excretion of human subjects during control, thiamine deprivation and low-level repletion

Subject	Day of study										
	Control		Depletion					Repletion			
	3	9	15	21	27	33	39	42	45	48	51
	μg/24 hours		*μg/24 hours*					*μg/24 hours*			
A	263	263	38	20	*	*	*	*	*	*	9
B	213	307	8	20	*	*	*	10	*	*	*
C	38	218	5	15	*	*	*	7	3	5	5
D	229	336	6	8	*	*	*	7	*	*	13
E	122	340	24	16	*	*	*	9	5	*	10
F	213	268	*	*	*	*	*	9	*	*	*
G	386	374	11	14	*	*	*	10	*	6	6
H	307	158	16	13	*	*	*	9	*	*	*

* No detectable amount.

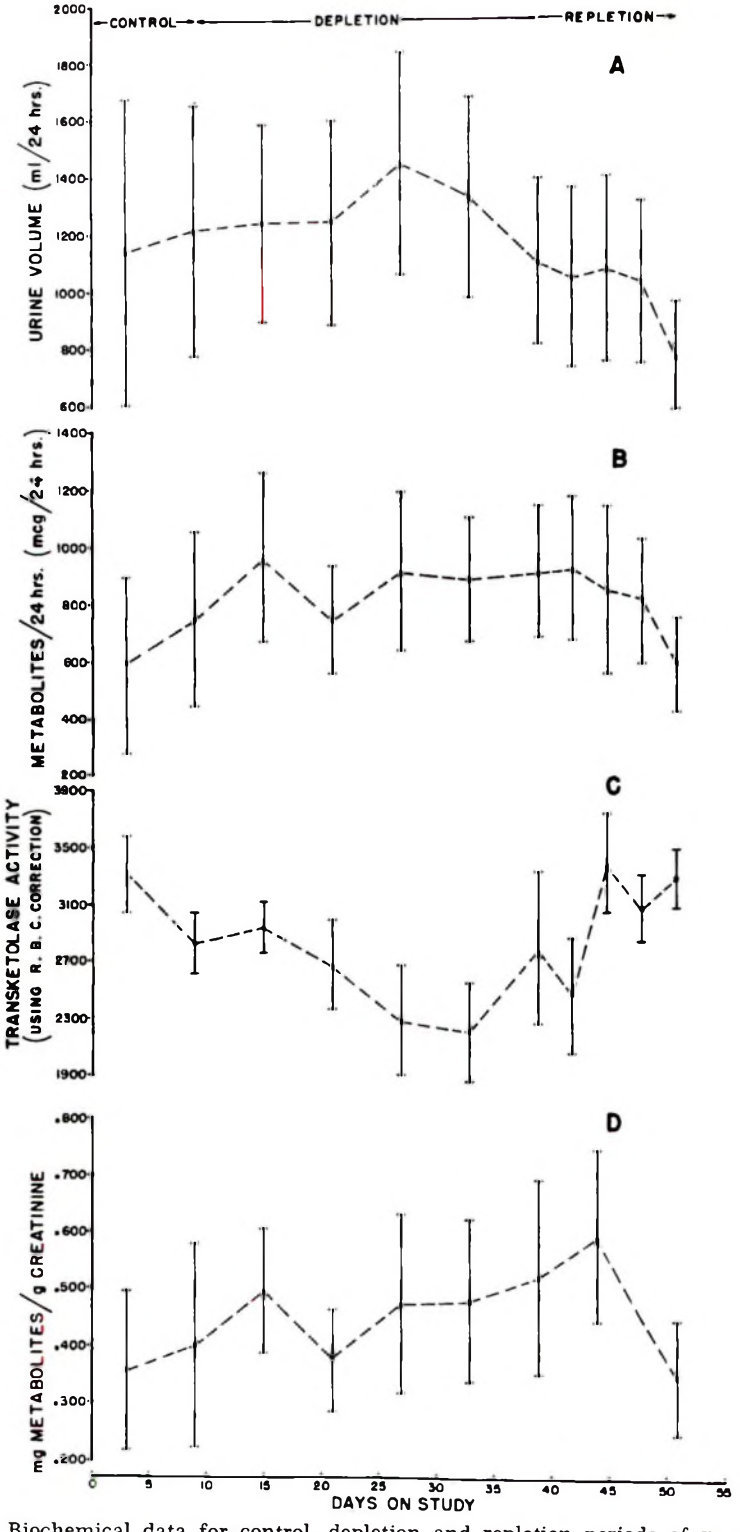

Fig. 1 Biochemical data for control, depletion and repletion periods of young men receiving restricted intakes of thiamine. A, urine volume (ml/24 hours); B, metabolites excreted (μg/24 hours); C, transketolase activity (using R.B.C. correction); and D, metabolites excreted (mg/g creatinine).

of the study (days 18 to 24 of depletion), the red blood cell transketolase levels were significantly different from those on day 9 ($P < 0.001$). However, a comparison between day 9 and day 30 of depletion showed no significant difference. This may be attributed to an abrupt increase in transketolase values which occurred simultaneously with an increase in the output of thiamine metabolites and an increase in the metabolite-to-creatinine ratio. After being repleted for six days at low levels (about 0.60 mg thiamine/day), the transketolase levels were significantly higher than the second control values ($P < 0.001$). At the end of the study with 12 days of low-level repletion, the transketolase values were significantly higher than those of period 2 during the control.

The ratio of milligrams of metabolites excreted to grams of creatinine excreted per 24 hours as a function of the control, depletion and repletion periods is plotted in figure 1(D). There is a distinct rise in the ratio so that on day 39 (depletion for 30 days) it was significantly higher than on day 9 (end of control period) ($P < 0.01$) and on day 44 (repletion for 5 days) also significantly higher than on day 9 ($P < 0.025$). A comparison between day 9 and day 51 (repletion for 12 days) shows no significant difference.

As the deficiency progressed, the subjects appeared to excrete larger amounts of the thiazole than of the pyrimidine moiety of thiamine (table 2). Since normal subjects excrete equal amounts of pyrimidine and thiazole, this indicates a destruction of the pyrimidine component in the thiamine-deficient individual. At the end of the study, 4 of the 8 subjects excreted amounts of pyrimidine that were equal to thiazole, possibly indicating a trend toward normal.

During the depletion period, there was a negative correlation ($P < 0.05$) between the urine volume and the concentration (μg/ml) of metabolites excreted.

There appeared to be no significant changes in the hematocrit and the blood values for cholesterol, nonesterified fatty acids and phospholipids of the subjects during depletion as compared with control and repletion (table 3). Urinary creatinine appeared not to change significantly throughout the study.

DISCUSSION

The validity of a urinary excretion test for thiamine status depends on a number of factors: 1) the renal factors involved in the excretion of thiamine and its metabolites; 2) the extent to which recent intakes of the vitamin affect urinary excretion levels; 3) the contribution of bacterially synthesized thiamine in the intestine to the body; and 4) the effect of other factors not related to dietary intake or to those above.

While there is no knowledge at present with respect to the manner in which the kidney handles thiamine and its metabolites during periods of thiamine deficiency, recent work with dogs (10–13) has shown

TABLE 2

Relation of pyrimidine and thiazole excretion during control, thiamine deprivation and low-level repletion

Subject	Day of study										
	Control		Depletion					Repletion			
	3	9	15	21	27	33	39	42	45	48	51
A		=[1]	P[2]	P	P	P	P	P	P	P	P
B		=	P	P	P	P	P	P	P	P	=
C		=	=	P	P	P	P	P	=	P	P
D		=	P	P	P	P	=	P	P	P	P
E		=	P	no value	P	P	P	P	=	P	=
F		=	P	=	P	P	P	P	P	P	P
G		=	P	=	P	P	P	=	P	P	=
H		=	P	P	P	P	P	P	P	P	=

[1] = indicates pyrimidine excretion equals that of thiazole excretion.
[2] P indicates pyrimidine excretion less than that of thiazole excretion.

TABLE 3

Mean values of various biochemical parameters measured during control, thiamine deprivation and low-level repletion

	Day of study										
	Control		Depletion					Repletion			
	3	9	15	21	27	33	39	42	45	48	51
Metabolites, μg/24 hrs	588 ± 306 [1]	748 ± 303	965 ± 292	747 ± 184	912 ± 275	884 ± 217	913 ± 224	923 ± 248	849 ± 288	807 ± 212	585 ± 161
Creatinine, g/24 hrs	1.60 ± 0.31	1.88 ± 0.40	1.93 ± 0.37	1.99 ± 0.25	1.96 ± 0.38	1.88 ± 0.31	1.84 ± 0.33		1.71 ± 0.22		1.69 ± 0.18
Serum cholesterol, mg/100 ml	168.3 ± 7.8	161.9 ± 9.7	164.2 ± 9.2	163.9 ± 15.0	170.2 ± 7.8	166.0 ± 10.1	159.5 ± 12.0		156.4 ± 14.1		155.8 ± 11.5
Nonesterified fatty acids, μEq/l plasma	442 ± 28	436 ± 90	380 ± 35	404 ± 25	420 ± 45	414 ± 84	350 ± 44		426 ± 55		398 ± 41
Phospholipids, mg/100 ml plasma	7.68 ± 0.74	7.62 ± 0.46	7.39 ± 0.36	7.62 ± 1.03	8.30 ± 0.82	7.77 ± 0.72	7.81 ± 0.72		7.50 ± 0.72		7.90 ± 0.63
Hematocrit	49.8 ± 1.5		50.1 ± 1.5	49.8 ± 1.7	49.9 ± 1.7	49.9 ± 2.1	48.2 ± 1.1		49.1 ± 2.1		48.3 ± 2.1

[1] Mean ± SD.

that the kidney actively excretes thiamine through the renal tubules with no destruction of the vitamin during its passage. Haugen (13) demonstrated that when tubular excretion was blocked by the use of probenecid, the amount of thiamine in the urine was decreased. Since blood contains free and phosphorylated thiamine, whereas the urine contains only free thiamine, it appears that thiamine pyrophosphate is actively dephosphorylated in the kidney or that the phosphorylated vitamin does not pass through the kidneys. Magyar (14) and Najjar and Holt (15) also reported that the amount of thiamine appearing in the urine was influenced by the status of renal function. Thus, in patients with kidney disease, the latter authors reported a diminished excretion of the vitamin. No work is available regarding the manner by which the kidney excretes the metabolites of thiamine.

Criticism has been leveled at the use of the urinary excretion test as a measure of thiamine status since a single determination may be influenced by recent intakes of thiamine and not accurately reflect the extent of saturation of the tissue, or the amount of thiamine available for body functions. Berryman et al. (16), Mickelsen and co-workers (17), Oldham and co-workers (18) and Pollack and co-workers (19) emphasize the fact that urinary thiamine levels are affected not only by recent intakes, but also by individual differences in the amounts of thiamine excreted when equal amounts are ingested. These criticisms would require, therefore, that other tests be applied along with the amount of excreted thiamine before establishing a judgment regarding the thiamine status.

Fecal thiamine derived from bacterial synthesis does not appear to be an important source for the vitamin since the excretion remained high even during restricted intakes (20, 21), and urinary output was little affected when complete or partial inhibition of coliform and other aerobic organisms was achieved through oral administration of streptomycin (22). Friedemann and co-workers (23) have shown that there is a very limited capacity for absorption of thiamine from the intestine when they established that an amount exceeding 2 mg/meal or 5 mg/day would cause an increase in the amount of fecal thiamine.

Factors other than dietary intake may influence the excretion of thiamine. Thus, age (24–26), physical state (27, 28), achlorhydria (29, 30), environmental temperature (31), physical exercise (32), and the administration of compounds such as α-tocopherol (33), digitalis, folliculin and vitamin D (34) are reported to affect the amount of thiamine excreted in the urine.

Despite the large number of factors other than dietary which may influence the amount of thiamine excreted in the urine, there is ample proof to establish that, other things being equal, a deficiency of thiamine produces a consistent pattern of excretory products related to thiamine. The present work adds another facet to this pattern by establishing the levels of urinary excretion for the pyrimidine and thiazole moieties of thiamine and provides simultaneous values for red blood cell transketolase. The pattern may be described as follows:

1. There is a very rapid decrease in the excretion of thiamine, reaching less than 50 μg/24 hours at the sixth day of depletion with no detectable amounts excreted by day 18 of depletion. These results are in agreement with those of Jolliffe et al. (35), Keys et al. (36), Melnick ('37), Williams and co-workers (38) and Pollack and co-workers (39). This has been interpreted as an indication that there are no body stores for thiamine since low intakes rapidly reduce the excretory levels to zero. However, as will be shown later, this is not true since the thiamine observed in the urine may be considered as "spillover" thiamine that is not utilized by the body and would therefore not accurately reflect body stores of the vitamin.

2. It is valid to use the retention of a single dose of thiamine as an index of thiamine saturation of the tissues (38, 40–42). Thus, the administration of 1 to 5 mg of thiamine per os, or intramuscularly, will result in a smaller percentage excretion of the dose in deficient individuals as compared with normals. Some have used 3- to 4-hour urine samples after administering the dose (15, 37, 43–45), while others favor a 24-hour collection (38, 41, 46, 47). It appears that some consider the excre-

tion of less than 7% of the administered dose in 24 hours as indicative of deficiency, although others accept less than 14% (46) or less than 20% (48) as evidence of deficiency. The fact that there is a lag in the appearance of thiamine in the urine of a repleted individual emphasizes the necessity for building up the body stores and explains our observation that at low-level repletion doses there was little, if any, thiamine appearing in the urine even after 12 days of repletion. Similar results have been reported (37, 38, 46, 49).

3. The rapid disappearance of thiamine from the urine of individuals receiving a deficient intake is not associated with a similar lowering of the excretion of the pyrimidine moiety of thiamine (19, 50). Our studies confirm these results while extending the metabolite picture to include a simultaneous measurement of the thiazole portion of the thiamine molecule. With a normal intake of thiamine (> 1 mg/day) the urine would be expected to contain approximately 220 to 350 μg of thiamine/24 hours, with a metabolite level 2 to 4 times that of the thiamine. As deficiency progresses, with thiamine decreasing, the metabolites stabilize at around 900 μg/24 hours for a 2800-kcal diet with a composition as previously described. From this new knowledge, which includes a measurement of the thiazole, it is observed (table 2) that the pyrimidine is further destroyed in the deficient individual, whereas the thiazole appears not to be destroyed. However, the work of Borsook et al. (51) and of Iacono and Johnson (52) points to considerable amounts of destruction products of thiazole following the administration of radioactively labeled compound. The present study permits the conclusion that in thiamine deficiency more of the pyrimidine is destroyed than in the normal individual.

These data imply that there is a body reservoir for thiamine which is called upon during times of deficiency. The drain on this reservoir may continue until a minimal threshold is reached beyond which the organism dies. The metabolites which appear in the urine represent the utilization of thiamine since ingested pyrimidine does not appear to be an important factor in contributing to that appearing in the urine (53).

4. The observation that a deficiency of thiamine produced a decreased activity in the metabolism of glucose via the pentose shunt pathway has been well documented (1, 54–56). This decrease has been proved to be due to a loss in transketolase activity. The correlation in this study between the progressive development of a thiamine deficiency and the decreased activity of the enzyme transketolase is confirmed, thus supporting the validity of the transketolase measurement as an index of thiamine deficiency. As reported by Brin (57), a period of 13 days was required for the thiamine deficiency to manifest itself in a reduction of transketolase activity of rat erythrocytes. In the present study with humans, the effect was noted at 12 days which is in agreement with the observations of Brin (58). It must be pointed out, however, that Brin used a semi-purified diet in his animal studies, presumably containing no thiamine, whereas the diet in the human study reported here contained 0.11 to 0.18 mg thiamine/day. The diet in Brin's human studies contained 0.19 mg of thiamine/day (58).

It appears that the transketolase activity measures the amount of thiamine pyrophosphate in the red blood cell. Florijn and Smits (59) reported that with a thiamine-deficient diet the decrease in red blood cell thiamine pyrophosphate paralleled the decrease in liver, kidney and muscle, thus validating the use of the red blood cells as an index of thiamine levels in the tissues. The metabolites excreted in the urine may be viewed as indicators of the thiamine that has been utilized, whereas the transketolase assay measures the amount of thiamine in the blood and is related to the amount of thiamine in such tissues as liver, kidney and muscle. Thus, while both measure the vitamin in different media or tissues, they each indicate different aspects of thiamine status. On day 30 of depletion (day 39, fig. 1(C) and (D)) there was an abrupt rise in transketolase activity with an increase in the metabolite-to-creatinine ratio, indicating an increase in available thiamine. Liang (60), working with thiamine-deficient rats, showed a decrease in pyruvate and α-keto

acid excretion at the twenty-fifth to twenty-seventh day also indicating an increase in available thiamine. Further depletion caused these latter substances to increase again until they decreased prior to death. Further work will be needed to determine whether these observations may be a consistent part of the picture in thiamine deficiency.

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Thiamine Requirement in the Adult Human as Measured by Urinary Excretion of Thiamine Metabolites [1]

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ABSTRACT Normal, healthy young men consumed a diet with a restricted intake of thiamine. Urinary excretion of thiamine, as well as the pyrimidine and thiazole moieties of thiamine, were measured. After 6 days of thiamine deprivation, the urinary thiamine excretion decreased to an average of 14 μg/24 hours. By the eighteenth day of deprivation, there was no detectable thiamine in the urine. Metabolite excretion increased during the depletion period and appeared to reach a constant level. It is proposed that this level of metabolite output represents a measure of the rate at which body stores of thiamine are being depleted. The maintenance of thiamine balance would require the amount that would replace the vitamin used for metabolic needs, and would represent the minimum daily requirement. The metabolite excretion from the eighteenth to the thirtieth day of depletion, when no thiamine was being excreted, averaged 903 μg/24 hours. However, the maximal amount excreted in any one day during this period was 913 μg/day. Two methods for calculating the thiamine requirement are possible: 1) subtracting the 160 μg of ingested thiamine from the metabolite output, leaving a net deficit of 753 μg of thiamine/day; this would make the minimum daily requirement 0.27 mg/1000 kcal; and 2) not subtracting the amount of ingested thiamine from the metabolite output, leaving a net deficit of 913 μg of thiamine/day. The minimum daily requirement would then be 0.33 mg/1000 kcal. Using a safety factor, the recommended daily allowance should be 0.54 to 0.66 mg/1000 kcal. This is in good agreement with previous work which correlated clinical symptoms with the available biochemical tests.

In attempting to assess the thiamine requirement of the human, many studies have approached the problem with a variety of experimental designs. In general, diets providing varying levels of thiamine were fed for specific periods of time while the subjects were observed and appropriate tests were applied. Thus, the intake of the vitamin was controlled, urinary and fecal excretions were measured and, in some cases, mental efficiency, work efficiency and performance, psychomotor tests, intellective tests and many other criteria were applied and evaluated. As a result, a close correlation was established between the development of a thiamine deficiency and the decreasing excretion of thiamine in the urine (1–10). But, as reported previously (11), the use of urinary excretion levels was criticized when it was evident that these levels were influenced by recent intakes of the vitamin and were therefore not an accurate reflection of thiamine status in the individual. More recently, experience has shown that the activity of blood transketolase also decreased as the thiamine deficiency progressed (11–13). However, while these methods have proved adequate for the detection of a developing and impending deficiency of thiamine, they were subject to error since they depended in the final analysis on a correlation between the biochemical values and the appearance of clinical deficiency symptoms. Since the appearance of these symptoms is often slow and gradual, it is difficult for the clinician to establish the point at which the patient is considered to be sufficient in

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[1] Unless otherwise specified, the word "metabolite" referred to in this paper signifies those compounds excreted in the urine which, when incubated with dried active baker's yeast (2), form a compound which behaves like thiamine in the USP or Association of Official Agricultural Chemists' fluorometric assay for thiamine.

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thiamine and beyond which he is deficient. These difficulties have prevented the precise definition of the deficient state in terms of urine thiamine and blood transketolase.

Another criterion that has been applied and shown to correlate well with the existence of a thiamine deficiency is the retention dose. It has been shown that a deficient individual will retain a greater percentage of an administered test dose of the vitamin (14–17). But here again, the establishment of a level of retention which correlated with deficiency suffered from an accurate determination of the point at which a deficiency was deemed to exist. Other symptoms such as nausea, emesis, anorexia, headache, which may be present during the period of thiamine restriction, are too nonspecific to be used as criteria for establishing the onset of deficiency. In view of these difficulties, it is unexpected to find that the reported minimum daily requirement for thiamine has fallen within a relatively narrow range. Keys et al. (18) concluded that 0.23 mg/1000 kcal is adequate, while Daum et al. (19) reported that 0.25 to 0.30 mg/1000 kcal was required. Most investigators have favored values higher than these: 0.31 to 0.35 mg/1000 kcal (3, 5, 20), 0.36 to 0.40 mg/1000 kcal (2, 4, 21), 0.41 to 0.45 mg/1000 kcal (2, 22), 0.46 to 0.50 mg/1000 kcal (23). Williams et al. (7) concluded that the minimal intake was somewhere between 0.22 and 0.50 mg/1000 kcal.

As a result of the development of a new method (24) which made possible the quantitative measurement of the metabolites of thiamine in urine, a new criterion was made available by which to measure the requirement for thiamine. This criterion does not require an estimation of the point of onset of thiamine deficiency, and therefore does not suffer from the criticisms leveled at the use of urinary excretion, red blood cell transketolase, and dose retention for the estimation of thiamine status. The work reported here will support the view that thiamine observed in the urine represents unused or "spill-over" thiamine; that during periods of restricted thiamine intake, when the thiamine in the urine is no longer detectable, the metabolites appearing in the urine are an index of the amount of thiamine taken from the tissue stores and utilized for metabolic needs.

EXPERIMENTAL

As reported in a previous paper (11), 8 healthy, young adult males were given diets providing an average of 2800 kcal with 0.11, 0.16 and 0.18 mg thiamine/day, depending upon the menu, which was rotated to provide some variety. During the control period, lasting 9 days, the diet was supplemented with 1.6 mg of thiamine. The depletion period of 30 days was divided into five 6-day periods, during which time the amount of thiamine available was that provided by the diet. The repletion supplement of 0.43 mg of thiamine was designed to test the effects of low-level repletion on the disappearance of the clinical complaints and symptoms developed during the depletion period. The repletion period lasted the scheduled 12 days, although a longer period at a higher level would have been more desirable.

The medical examinations and physical evaluations were carried out as described previously (11), as were the collection of samples and their analyses for thiamine and its metabolites.

RESULTS

The values for excretion of thiamine and thiamine metabolites for the individual subjects are presented in table 1. On the last day of the control period (table 2), the 8 subjects excreted in their urine an average of 283 μg of thiamine/24 hours. Six days after the depletion began, the 8 subjects excreted an average of 14 μg of thiamine/24 hours, whereas after 12 days of depletion they excreted an average of 13 μg of thiamine/24 hours. However, on day 18 of depletion, no detectable amount of thiamine was excreted by any of the subjects. During these same periods the metabolite excretion increased, but not to a significant extent. Metabolite excretion on day 18 of depletion was significantly higher than that on the last day of the control period ($P < 0.025$). Despite the failure of metabolite excretion in control versus depletion periods to achieve

TABLE 1

The excretion of thiamine and thiamine metabolites of human subjects during control, thiamine deprivation and low-level repletion

Subject		Day of study										
		Control		Depletion					Repletion			
		3	9	15	21	27	33	39	42	45	48	51
		μg/24 hours		*μg/24 hours*					*μg/24 hours*			
A	Thiamine	263	263	38	20	*	*	*	*	*	*	9
	Metabolites	339	1232	1110	778	1374	877	1001	1238	1256	743	854
B	Thiamine	213	307	8	20	*	*	*	10	*	*	*
	Metabolites	696	779	946	759	1134	1001	1037	1351	671	946	429
C	Thiamine	38	218	5	15	*	*	*	7	3	5	5
	Metabolites	241	358	625	638	636	483	872	819	509	472	634
D	Thiamine	229	336	6	8	*	*	*	7	*	*	13
	Metabolites	757	744	708	628	679	1125	722	689	808	599	402
E	Thiamine	122	340	24	16	*	*	*	9	5	*	10
	Metabolites	284	326	686	553	605	970	783	728	539	699	488
F	Thiamine	213	268	*	*	*	*	*	9	*	*	*
	Metabolites	467	732	1034	1038	892	697	835	877	780	961	752
G	Thiamine	386	374	11	14	*	*	*	10	*	6	6
	Metabolites	807	787	1505	588	865	809	679	722	976	1087	636
H	Thiamine	307	158	16	13	*	*	*	9	*	*	*
	Metabolites	1114	1023	1106	996	1112	1108	1375	960	1243	951	490

* No detectable amount excreted.

TABLE 2

Mean excretion of thiamine and thiamine metabolites of human subjects during control, thiamine depletion and repletion periods

	Day of study										
	Control		Depletion					Repletion			
	3	9	15	21	27	33	39	42	45	48	51
	μg/24 hours				*μg/24 hours*					*μg/24 hours*	
Thiamine	221 ± 107 [1]	283 ± 71	14 ± 12	13 ± 7	*	*	*	8 ± 3	1 ± 2	1 ± 3	26 ± 5
Thiamine metabolites	588 ± 306	748 ± 303	965 ± 292	747 ± 184	912 ± 275	884 ± 217	913 ± 224	923 ± 248	849 ± 288	807 ± 212	585 ± 161

[1] Mean ± SD.

* No detectable amount excreted.

differences that were consistently significant, the numerical values were higher during depletion than during the control period. A comparison of metabolite excretion between the fifth and twelfth day of repletion shows the latter to be significantly lower ($P < 0.01$), reaching a value that is equal to that obtained during the control period.

During the first 2 days of repletion, the subjects excreted 6 to 10 μg of thiamine each day. From the third to the ninth day, barely detectable amounts were observed in no more than 2 subjects each day, with no consistent output by any one subject. On the tenth to twelfth days, the subjects excreted from 4 to 18 μg of thiamine.

DISCUSSION

The rapid decrease in the urinary excretion of thiamine of subjects receiving restricted intakes supports the view that thiamine observed in the urine of well-fed individuals represents that portion of the vitamin that is in excess of the body's needs. Nevertheless, during the time that thiamine is in excess and is being excreted, there is also a considerable amount of pyrimidine and thiazole appearing in the urine. Mickelsen et al. (10) attempted to establish a quantitative relationship between thiamine and its pyrimidine component. They showed that with dietary intakes ranging from 0.6 mg to 2.0 mg of thiamine/day, the amount of thiamine in the urine was linearly related to intake, although one individual might excrete 2 or 3 times as much as another receiving the same diet. When these authors measured the pyrimidine component of thiamine in the urine, they found an exponential relationship to thiamine at high intakes of the vitamin, with an almost linear relation at an intake of 1 to 2 mg daily. As a result of these studies and others, it appears difficult to establish an understanding of the factors which govern the relationship between thiamine and its metabolites excreted in the urine. Of importance in these considerations is the fact that at low levels of thiamine intake, when thiamine no longer appears in the urine, the metabolites continue to be excreted to an even greater extent than when thiamine is present.

The problem with respect to the source of the metabolites during periods of low intakes of thiamine must be considered before an evaluation can be made of the extent to which these compounds may be related to the metabolism of thiamine. If Najjar and Holt (25) are correct, intestinal synthesis of thiamine contributes to the body's needs when the vitamin is absorbed across the wall of the large intestine. Others, however, disagree that thiamine synthesis by intestinal microorganisms is important in man (26–29). Alexander and Landwehr (22) have shown that 50 mg of crystalline thiamine administered as a retention enema and kept for 24 hours did not significantly elevate the amount of thiamine excreted in the urine of the subject. Also, despite the presence of significant quantities of thiamine and thiamine pyrophosphate in the fecal matter of the intestine, the preponderance of evidence would make it appear that this vitamin is not available to the individual.

Caster and Mickelsen (30) have investigated the possibility that the pyrimidine which appears in the urine might be of exogenous origin, such as that derived from food. When they fed young men up to 4 mg of this component and measured it in the urine, they calculated that only 3% was absorbed and excreted as such. They also observed that this dose of pyrimidine did not affect the amount of thiamine excreted in the urine, indicating that the pyrimidine was not utilized by the body for the synthesis of the vitamin. Thus, it appears that pyrimidine and thiamine observed in the urine are not derived from ingested pyrimidine.

It is pertinent to question whether the substances reported as metabolites in urine might not be derived from other pyrimidine and thiazole compounds in the body which could replace the thiamine pyrimidine and thiazole moieties in the yeast resynthesis of thiamine. Williams et al. (31) have compiled the work of many authors who used 22 pyrimidine analogues and 25 thiazole analogues, and have concluded that "the thiamine molecule can undergo very little modification without extensive loss of vitamin B_1 activity." They further state that "for many microorganisms the structural specificity

of vitamin B_1 is substantially the same as for animals." There is thus good reason for believing that the pyrimidine and thiazole which appear in the urine of man and which can be utilized by yeast for the resynthesis of thiamine were very likely derived from ingested thiamine. This does not rule out the fact that other metabolites of thiamine may exist as reported by Iacono and Johnson (32), Borsook et al. (33) and Neal and Pearson.[4] However, the quantitative relationship between the degradation products reported by these authors and the amount of ingested thiamine remains to be established. The extent to which these must be considered to obtain a more accurate picture of thiamine metabolism is not yet known.

From the fact that pyrimidine derived from exogenous sources does not significantly influence the amount of this substance in the urine, and that fecal thiamine is not available to the host, it appears that the metabolites of thiamine excreted by the body are quantitatively related to the amount of ingested thiamine. The continued excretion of these metabolites during periods of restricted intake of thiamine can only mean that the stores of the vitamin in the tissues are being utilized for metabolic purposes. Thus, these excreted compounds, which may be converted quantitatively to thiamine equivalents, represent a measure of the extent to which these stores are being depleted. We may then view the thiamine requirement as that amount which would at least equal the amount of metabolites excreted when there is no intact thiamine in the urine. When this amount is provided, the subject would be in thiamine balance; his thiamine intake would just equal his metabolite excretion.

Data presented in this paper show that beginning with the eighteenth day of depletion, no detectable amount of thiamine was excreted in the urine. From then to day 30 of depletion the metabolite excretion ranged from 884 to 913 μg /24 hours. Subtracting the 160 μg of thiamine ingested in the diet, the net deficit, using 913 μg as the amount excreted, would be 753 μg/24 hours. In order to maintain the subjects in balance, they would have required at least 0.27 mg of thiamine/1000 kcal. This may be viewed as the minimum daily requirement. Allowing for the safety factors which compensate for variations in individual requirements, cooking losses, etc., an individual should have 0.54 mg of thiamine/1000 kcal of diet assuming the usual distribution of calories from protein, carbohydrate and fat in the diet. These data are in excellent agreement with those of Mickelsen et al. (10) who reported that when the thiamine intake fell below 0.7 mg/day, the thiamine excretion approached zero.

An alternate method of calculation may be considered. It is possible that low intakes of thiamine do not alter the quantities of metabolites that are excreted in the urine. Thus, increasing the intake of thiamine, say from 160 μg to 300 μg, might not increase the metabolite output by 140 μg. This is supported by the observation that during repletion, as at day 42 of the study (11) (table 3), where the intake was 600 μg/day, the metabolite excretion was not significantly different from that during the depletion period where the intake was 160 μg/day. There would then be no justification for the subtraction of the ingested thiamine from the excreted metabolites in arriving at a measure of the body's requirement for thiamine. In this event, the excretion of 913 μg of metabolites with a 2800-kcal diet would require 0.33 mg of thiamine/1000 kcal to balance the output. With this as the minimum daily requirement, the recommended daily allowance should be 0.66 mg/1000 kcal. It would be necessary to measure the metabolite output of humans with a zero thiamine intake before reaching a conclusion as to the validity of each method of calculation.

The value of such a concept lies in the fact that there is a measurable point at which the deficiency may be said to begin, namely, when the metabolite and thiamine excretion exceeds the thiamine intake. This eliminates the dependence upon the slow appearance and relatively inaccurate determination of the clinical syndrome. When coupled with the red blood cell transketolase activity, which measures thi-

[4] Neal, R. A., and W. N. Pearson 1964 Identification of a new metabolite of thiamine in mammalian urine. Federation Proc., 23: 242 (abstract).

amine in the blood, and the retention, which measures the degree of unsaturation of the tissues, meaningful biochemical tests are now available which may be used to estimate the thiamine status of an individual.

It is emphasized that the studies reported here were performed with healthy, young adult males on a schedule of moderate activity, receiving a restricted intake of thiamine and maintained with a diet with the usual proportion of calories from carbohydrate, fat and protein. Their intake of known water-soluble and fat-soluble vitamins was assumed to be adequate because of the supplementation. The metabolite excretion patterns of the young, the aged, the beriberi patient, the individual with a marginal intake of thiamine, and the female, both pregnant and nonpregnant, as well as lactating, remain to be studied.

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Chemical Characterization of Inbred-strain Mouse Milk

I. GROSS COMPOSITION AND AMINO ACID ANALYSIS

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ABSTRACT Milk from 2 unrelated inbred strains of mice, DBA/2J and C57BL/6J, was found to differ both qualitatively and quantitatively with respect to the amino acid composition. These differences were evident in the unhydrolyzed free amino acid fraction. Seventeen amino acids were observed in the milk of both strains, but in strain DBA/2J several were present in two to five times the amount observed in the milk of strain C57BL/6J. Methionine and lysine occurred in substantial amounts in strain DBA/2J milk, but only in traces in the milk of strain C57BL/6J. Histidine was virtually lacking in the milk of DBA/2J, but present in that of strain C57BL/6J. The results of this study are requisite to the development of a diet suitable for artificial feeding of gnotobiotic mice.

Milk was known to be a complex substance even before the more recent progress in analytical techniques. Milk from various species, although similar in many respects, differs in composition both qualitatively and quantitatively. These differences may be significant in the utilization of milk from one species by another. Also, they reflect different nutritive requirements of the young of each species, and perhaps too, may be of significance in the interrelated biochemical and nutritional roles of certain constituents. Yet, while no perfect substitute is available for milk, in the germfree rearing of several mammals (mostly larger species), neither colostrum nor natural milk was necessary. In contrast, great difficulty has been encountered in rearing rodents with artificial milk (see review (1)).

Our concern and interest in the composition of mouse milk stems from failure or only sporadic success in rearing germfree inbred mice. Of equal interest to us, was to investigate possible strain-specific differences in milk composition since it is well known that inbred strains of mice differ considerably in their growth characteristics. The present paper reports on certain gross compositional observations in mouse milk and presents complete amino acid analyses for milk of 2 strains of mice.

EXPERIMENTAL

Animals. The 2 inbred strains studied were the C57BL/6J and the DBA/2J. The mice were fed diets identical in protein content (19%) and source (skim milk and wheat); the diet for strain C57BL/6J contained 11% fat, and that for DBA/2J was 8%, the source being corn oil. Fiber content was 2% in both feeds. A slightly lower content of fat for the DBA/2J strain was previously found to increase its reproductive capabilities (2). The diets were made up essentially according to the Morris formulations (3).

Milk samples. We have recently described a simple unit for milking mice (4); the apparatus is easily manipulated, sturdy and conveniently applied. It provides for rapid flow, thereby eliminating evaporation, an inherent major drawback in all other models. The mice were preconditioned by removal from their suckling young at least 12 hours prior to milking. Ten to twenty minutes before milking they were injected intravenously with 0.4 to 0.6 USP units purified oxytoxic principle/kg.[2] Thus, it was easily possible to collect from 0.5 to 2.4 ml of milk from lactating mice 8 to 14 days postpartum. For the various analyses, samples from individual mice and pools were used.

Electrophoresis. The procedure of Smithies (5) was used for both the starch gel preparation and separation; a gradient of 5 volts/cm was maintained for 16 hours.

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[2] P.O.P., Armour Pharmaceutical Company, Chicago.

Patterns of milk serum were compared with isologous (same strain) blood serum for reference purposes.

Chemical analysis. Schain test reagent [3] was used for determining total lipids. Since the lipid volume always exceeded the 50 calibrated units, the milk sample was diluted volumetrically with physiologic saline. Butterfat was measured by the Gerber test (6) that was adapted to a microprocedure for individual mouse milk samples.[4] Cholesterol, chloride and protein were determined with the Beckman Spinco microanalytical system. The percentage water content was obtained by weight difference of samples before and after evaporation. Complete amino acid analyses were performed by ion-exchange column chromatography.[5] Deproteinization for free amino acid analysis was effected by several volumes (5 to 10) of 1% picric acid; the picrate was then removed by ion-exchange chromatography prior to analysis. Hydrolysates were prepared employing the following conditions: 100 volumes of constant boiling HCl at 100° for 22 hours in a sealed-evacuated Pyrex tube. Approximately 15 ml of pooled milk were required for a complete amino acid analysis.

RESULTS

Gross composition and electrophoresis. Table 1 contains data on a number of gross constituents of milk; there was no difference between those for strains C57BL/6J and DBA/2J for any of the parameters measured and listed. To judge from either the low water content or the high percentage of solids, mouse milk is very concentrated. Particularly, a very high content of lipids is apparent. Butterfat is several times (3 to 7 ×) higher than in cow's milk. While lipids are vastly greater in amount, cholesterol is lower than in mouse serum, but approximately intermediate between that of human breast milk and cow's milk.

TABLE 1

Gross composition of milk from strain C57BL/6J mice

Water, %	38.7 ± 5.8(15) [1]
Solids, %	61.3(15)
Total lipids, %	41.6 ± 7.3(26)
Butterfat, %	22.0 ± 3.7(25)
Cholesterol, mg/100 ml	36.9 ± 11.8(10)
Protein, %	14.1 ± 1.6(10)
Chloride, mEq/liter	45.2 ± 4.7(7)

[1] Numbers in parentheses indicate number of individual determinations.

To study mouse milk proteins, comparisons were made of electrophoretic patterns between strain C57BL/6J milk and blood serum. While there were several bands in common and representing physico-chemically similar protein fractions, there were also some that were specific for mouse milk. These are lactalbumin and casein, but in addition certain others were present, the nature and function of which are as yet unknown. Total proteins were also much higher than in milk of cows, goats, sheep or humans, but were roughly similar in amount to that observed in mouse blood plasma.

Amino acid composition. The total amount of protein was slightly higher in milk samples from strain C57BL/6J mice (14.10%) than from mice of the DBA/2J strain (11.10%). The ammonia content for the milk hydrolysates of both strains was almost identical, 0.31% (DBA/2J) and 0.33% (C57BL/6J), respectively; however, there was approximately one quarter more in the unhydrolyzed DBA/2J strain milk (12.5 mg/100 ml) than in that of strain C57BL/6J (9.12 mg/100 ml). The total amount of free amino acids in strain DBA/2J milk was twice that of the C57BL/6J strain, 18.61 mg/100 ml and 9.32 mg/100 ml, respectively. A complete amino acid analysis is presented in table 2. The identical 17 amino acids occur in both strains, but the unhydrolyzed portion of DBA/2J strain milk contains four in trace quantities (less than 0.02 mg/100 ml), whereas in C57BL/6J strain milk five are present as traces only. Half-cystine, phenylalanine and tyrosine occur as only traces in both strains. Methionine and lysine in substantial amounts are contained in DBA/2J strain milk but are essentially absent from milk of the C57BL/6J strain, whereas histidine is virtually lacking in strain DBA/2J milk but present in that of strain C57BL/6J. Several amino acids, glutamic acid, proline, glycine, valine, methionine, leucine, lysine and

[3] Lipitest Reagent, Merck and Company, Inc., Rahway, New Jersey.

[4] Allen, R. C., unpublished data, 1963.

[5] Analytica Corporation, New York.

TABLE 2

Amino acid analysis of milk from 2 strains of mice

Amino acids	Milk hydrolysate		Unhydrolyzed milk	
	Strain DBA/2J	Strain C57BL/6J	Strain DBA/2J	Strain C57BL/6J
	g/100 ml	*g/100 ml*	*mg/100 ml*	*mg/100 ml*
Aspartic acid	0.93	0.97	0.40	0.39
Threonine	0.54	0.57	0.49	0.44
Serine	0.87	0.95	1.56	1.23
Glutamic acid	1.98	2.38	4.57	2.12
Proline	0.89	1.03	2.91	1.70
Glycine	0.18	0.17	1.71	0.93
Alanine	0.49	0.56	0.70	0.75
½ Cystine	0.23	0.29	tr [1]	tr
Valine	0.62	0.71	1.61	0.84
Methionine	0.38	0.41	0.37	tr
Isoleucine	0.54	0.55	0.24	0.14
Leucine	1.11	1.14	0.91	0.16
Tyrosine	0.40	0.46	tr	tr
Phenylalanine	0.53	0.57	tr	tr
Lysine	0.74	0.76	1.98	tr
Histidine	0.30	0.33	tr	0.14
Arginine	0.37	0.38	1.16	0.28

[1] Trace indicates less than 0.02 mg/100 ml.

TABLE 3

Total amino acid composition of mature milk from 3 species

Amino acid	Rat	Cow	Man	
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	
Aspartic acid	50.5 [1]	166 [2]	116 [2]	—
Threonine	39.1	152	54	51.8 [3]
Serine	—	160	69	—
Glutamic acid	175.5	680	230	—
Proline	67.9	250	80	—
Glycine	14.8	11	0	—
Alanine	—	75	35	—
½ Cystine	34.8	23	20	—
Valine	43.5	239	71	72.5
Methionine	16.5	71	14	11.6
Isoleucine	40.9	218	68	61.0
Leucine	49.8	312	108	96.6
Tyrosine	33.9	170	50	—
Phenylalanine	33.1	153	41	40.4
Lysine	46.1	256	76	70.1
Histidine	21.7	92	23	23.7
Arginine	29.6	133	45	43.3
Tryptophan	40.0	49	18	19.2

[1] Values converted from percentage of protein (8.7 g/100 ml), from Luckey (9).
[2] Values from Altman and Dittmer (8).
[3] Available values from Macy (10).

arginine are elevated approximately 2 to 5 times in unhydrolyzed milk of strain DBA/2J as compared with that of strain C57BL/6J. For comparison with our data, the total amino acid composition of milk from three other species is presented in table 3.

DISCUSSION

Analysis of mouse milk is prerequisite to the development of a diet suitable for artificial feeding and rearing of neonatal or Caesarean-derived mice. No previous investigations have been made of the composition of mouse milk owing, apparently, to the difficulty of obtaining a large enough sample for analysis.

Inspection of the gross composition of mouse milk (table 1) shows that it is very concentrated compared with that of dairy cows which contains roughly 88%

water, and with that of other rodents, namely, rat's milk, with a water content of around 74% (7–9). The only other mammals known to produce milk of such low water content are certain marine mammals (seals and whales) and the kangaroo rat. In these, the amount of fat in the milk is also very high, in fact even higher than in mouse milk (7). It has been speculated that a diet containing a high proportion of fat might be advantageous to both mother and young for water conservation. Analyses for major and minor constituents including minerals and vitamins, are continuing. Since differences in amino acids, both free and bound, of tissues and fluids have been observed in a variety of species, including man (6), it seemed worthwhile to study the amino acid patterns of the milk of two unrelated inbred strains of mice. As both strains were fed an essentially similar diet (except for a 3% difference in fat content) any significant deviations may be assumed to be due to their genotypic differences. Such differences, both qualitative and quantitative, exist regarding several amino acids of unhydrolyzed milk (see table 2). A genetic analysis to clarify a possible hereditary nature of the observed differences is in progress and consists intially of determining the free amino acid composition of milk from F_1-hybrids ($B6D2F_1$) and backcrosses to one or both of the parental strains. Also, studies on the lipid composition both regarding the types and relative quantities of saturated and unsaturated fatty acids are in progress. Comparison of the amino acid patterns from mouse milk, and milk from rats, cows and man, show a number of differences (see tables 2 and 3). For example, rat's milk apparently lacks serine and alanine, and human milk is devoid of glycine. The lack of tryptophan in the mouse milk analysis is the result of its destruction upon acid hydrolysis. Clearly, it must be a constituent of mouse milk, since it has been unequivocally demonstrated to be an essential amino acid for the mouse. With respect to the accuracy of the values listed for rat's milk, it is important to state that the analyses were made by microbiological assay of dialyzed, acetone-precipitated protein. The precision may be considerably less than that obtained by ion-exchange chromatography analysis.

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Response of Rats to Thalidomide as Affected by Riboflavin or Folic Acid Deficiency [1]

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ABSTRACT The response of rats to thalidomide was examined under the influence of either riboflavin or folic acid deficiencies. At a level of 0.75% of the diet thalidomide depressed growth, but this was significant only for rats fed by the equalized feeding procedure, and for rats fed the riboflavin-deficient diet, ad libitum. In the former case the growth-depressing effect of thalidomide was markedly intensified by omission of riboflavin from the diet. In contrast, a riboflavin depletion-repletion regimen showed a greater degree of recovery in rats receiving thalidomide. Inclusion of thalidomide in the riboflavin-deficient diet accentuated the typical symptoms and produced a consistent leucopenia. Thalidomide had little or no specific effect on liver content or activity of riboflavin-dependent enzymes and cofactors, such as D-amino acid oxidase, FAD and flavin-mononucleotide. However, succinic dehydrogenase, but not glutamic dehydrogenase, was decreased. Centrolobular necrosis was induced by thalidomide only in the livers of rats receiving the diets used in the folic acid study which contained sulfothalidine; thalidomide also depressed growth in all rats receiving the diets with sulfothalidine.

Several mechanisms of action have been postulated to explain the teratogenic activity of thalidomide. Roath et al. (1) suggested that thalidomide may behave as a glutamic acid antagonist. He based his suggestions on structural considerations and the fact that some established glutamic acid antagonists have been known to cause experimental teratogenesis. Evidence against this proposal has been offered by Narrod and King (2) and Hirschberg et al. (3), although Williams (4) demonstrated that some of the derivatives of thalidomide isolated from urine will inhibit brain glutamine synthetase and Rauen (5) reported that thalidomide slightly inhibits the growth of *Leuconostoc mesenteroides*, although this inhibition can be reversed by L-glutamic acid.

Kempner (6) suggested that thalidomide may act as a folic acid antagonist. Experimental support of this theory was cited by Nystrom (7) who showed that the concentration of formiminoglutamic acid is increased in the urine of cancer patients treated with thalidomide. Frank et al. (8), using a solubilized preparation of the drug, demonstrated that thalidomide interferes with the nicotinamide metabolism of protozoa. However, Narrod and King (2) were unable to produce teratogenic effects in rats with a similar preparation.

Patients who developed glossitis as a result of prolonged thaladomide administration responded to treatment with vitamin B complex (9). Failure of thalidomide to interfere with the transport of amino acids through gut membranes was used as evidence to refute the possibility that the drug acts as a vitamin B_6 antagonist (10).

Leck and Miller (11) have pointed out striking similarities between the embryonic malformations produced by riboflavin deficiency in rats and those induced in humans by thalidomide treatment. In both cases, skeletal rather than visceral malformations predominated. In addition, as these authors indicated, the structures of the vitamin and the drug are somewhat similar.

If thalidomide is a vitamin antagonist, its toxicity should be increased when it is administered to rats fed a diet deficient in the specific vitamin. In the present report we describe the response of rats to thalidomide added to diets deficient in riboflavin or folic acid. We observed that the growth of rats fed a riboflavin-deficient diet was markedly reduced by thalidomide, as compared with rats fed the supplemented diet.

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[1] A preliminary report of the experiments cited here has been presented at the 48th Annual Meeting of the Federation of American Societies for Experimental Biology, April 13, 1964.

However, we found no evidence based on metabolic studies that thalidomide functioned as a riboflavin antagonist.

EXPERIMENTAL

Table 1 shows the composition of the diets used for the folic acid and riboflavin studies. Thalidomide,[2] riboflavin, and folic acid, when fed, were included in the diets at levels indicated in the tables. The folic acid deficiency was induced by inclusion of a sulfa drug in the diet.

Weanling rats, weighing 40 to 55 g, of an Osborne-Mendel derived strain (Food and Drug Administration, Division of Nutrition) and a Sprague-Dawley strain (Charles River Farm) were used. Rats were fed either on an ad libitum or equalized feeding basis. In the latter, each rat was given 4 g of feed/day and allowed unlimited access to water. Weight gain was determined weekly.

This technique, which is a form of "paired feeding" in which the food consumption of individual animals of entire groups is restricted to the consumption of the poorest eater of any group, was used to insure that each animal would consume exactly the same amount of thalidomide.

TABLE 1

Composition of basal diets

	Riboflavin-deficient diet	Folic acid-deficient diet
	%	%
Sucrose	65	60
Casein, vitamin-free	20	18
Salts [1]	4	4
Cellulose [2]	—	4
Cottonseed oil, USP	8	—
Hydrogenated vegetable oil [3]	—	10
Cod liver oil, USP	2	—
Phthelylsulfathiazole [4]	—	2
Vitamins A, D, E, K [5]	—	1
Vitamin supplement 1 [6]	1	—
Vitamin supplement 2 [7]	—	1

[1] Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, 24: 245.

[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.

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

[4] Sulfathalidine, Sharp and Dohme, Philadelphia.

[5] Containing: (in mg) vitamin A, 6.67; vitamin D_3, 0.11; menadione, 25; DL-α-tocopherol, 25; ethoxyquin (Santoquin, Monsanto Chemical Co., St. Louis), 125; plus soybean oil to make 10 g.

[6] Containing: (in mg) thiamine·HCl, 5; pyridoxine·HCl, 6; Ca pantothenate, 25; nicotinamide, 20; D-biotin, 0.1; folic acid, 2; vitamin B_{12}, 0.2; inositol, 200; choline chloride, 2,000 plus sucrose to make 10 g.

[7] Containing: (in mg) thiamine·HCl, 10; pyridoxine·HCl, 10; Ca pantothenate, 30; nicotinamide, 50; D-biotin, 0.2; vitamin B_{12}, 0.03; inositol, 500; choline chloride, 1,000; riboflavin, 10; plus glucose monohydrate (Cerelose, Corn Products Co., Argo, Illinois) to make 10 g.

D-Amino acid oxidase (DAO) activity of liver homogenates was determined by the method of Robins et al. (12) as modified by Prosky[3] in which pyruvate, the end product of the reaction, is reacted with 3-hydrazinoquinoline to produce a compound which absorbs at 305 mμ. To tubes containing 0.2 ml of 0.25 M D-alanine in 0.05 M sodium pyrophosphate, pH 8.3, was added 0.02 ml of a 10% liver homogenate prepared in 0.2 M pyrophosphate, pH 8.3. The tubes were placed in a water bath at 38° and incubated for 15 minutes; then the reaction was stopped by the addition of 0.025 ml of 50% trichloroacetic acid. The tubes were centrifuged and 0.2 ml of the supernatant fluid was mixed with 0.2 ml of 0.5 mM 3-hydrazinoquinoline. After 15 minutes at 38° one milliliter of 0.01 M HCl was added to the tubes and the absorption at 305 mμ was measured. The appropriate blanks and pyruvate standards were carried through the entire procedure. Activity is expressed as micromoles of pyruvate produced per gram of liver per hour. Succinic-dehydrogenase (SDH) and glutamic dehydrogenase (GDH) were determined spectrophotometrically, essentially by the method of Slater et al. (13) in which the rate of reduction of ferricyanide is measured. Activity is expressed as the change in optical density at 400 mμ/g of liver/hour. Succinoxidase was assayed manometrically by adding 0.5 ml of a 10% liver homogenate to a Warburg flask which contained 40 μmoles of NaCl, 12 μmoles of KCl, 4 μmoles of $MgSO_4$ and 60 μmoles of succinate in a final volume of 3.0 ml. The center well of the flask contained 0.2 ml of 30% KOH, the gas phase was air, and oxygen uptake was determined over a 60-minute period. The results are expressed as micromoles of oxygen consumed per gram per hour. Plasma lactic acid dehydrogenase, alkaline phosphatase, and transaminase were de-

[2] Obtained from the William S. Merrill Company and found to be pure as determined by ultraviolet spectrophotometry.

[3] Personal communication, L. Prosky, Albert Einstein School of Medicine.

termined as described in several technical bulletins.[4,5,6]

The concentration of liver flavin coenzymes was determined by the fluorimetric assay of Burch (14), liver proteins by the method of Layne (15), and liver nuclei acids by the method of Schneider (16). Total liver lipids were assayed gravimetrically. Lipids were obtained by extracting an aliquot of liver homogenate twice with chloroform-ethanol, 1:2, and once with ether. Solvents were removed from the combined extracts and the residue was taken up in petroleum ether. Liver moisture was determined gravimetrically by difference after portions of the liver were dried under partial vacuum at 65° for 24 hours. Some of the livers were examined histologically by standard techniques using hematoxylin and eosin stain, and by the use of frozen sections. Formiminoglutamic acid determinations were performed on rat urine by the colorimetric method described by Tabor et al. (17). Blood cell counts and hemoglobin determinations were made by conventional techniques. Student's t test was used to test mean differences for significance ($P < 0.05$).

RESULTS AND DISCUSSION

Riboflavin studies. Thalidomide accentuated the symptoms of riboflavin deficiency, including poor growth, thinned fur and lesions of the face, leg and tail. Cumulative weekly weight changes of rats are presented in tables 2 and 3. All rats fed thalidomide averaged lower weight gain than their corresponding controls; however, it was only in the rats fed riboflavin-deficient diets that thalidomide exerted a consistently significant depressive effect. The magnitude of this effect was greater in rats restricted in their food intake than in those eating ad libitum (table 2). Food restriction caused death of about one-third of the riboflavin-deficient rats; thalidomide did not alter the mortality rate. Response of the Osborne-Mendel rats was similar in both males and females; in the Sprague-Dawley rats, however, thalidomide and riboflavin deficiency affected the females earlier than the males.

In a repletion experiment, a group of female and male Sprague-Dawley rats were fed riboflavin-deficient diets; one-half of

[4] Berger, L., and D. Broida 1962 The colorimetric determination of lactic dehydrogenase in serum or other fluids at 400 to 550 mμ. Sigma Technical Bulletin no. 500. Sigma Chemical Company, St. Louis.

[5] Sigma Chemical Company 1961 The colorimetric determination of phosphatase in serum or other fluids at 400 to 420 mμ. Sigma Technical Bulletin no. 104. Sigma Chemical Company, St. Louis.

[6] Sigma Chemical Company 1961 A simplified method for the colorimetric determination of glutamic-oxalacetic and glutamic pyruvic transaminases at approximately 505 mμ. Sigma Technical Bulletin no. 505. Sigma Chemical Company. St. Louis.

TABLE 2

Growth-depressing effect of thalidomide in male rats [1] as affected by riboflavin depletion

Riboflavin, mg/kg diet	0	0		6.2	6.2	
Thalidomide, g/kg diet	0	7.5		0	7.5	
		Cumulative weight gain				
days	*g*	*g*	*P value*	*g*	*g*	*P value*
		Equalized feeding (4 g/rat/day)				
7	4.0 ± 0.8 [2]	0.6 ± 0.8	0.05	5.2 ± 0.8	2.1 ± 0.8	0.10
14	6.4 ± 0.9	1.5 ± 1.0	0.01	12.8 ± 1.4	8.4 ± 1.2	0.05
21	6.9 ± 0.8	1.5 ± 0.9	0.01	17.4 ± 0.9	12.9 ± 1.3	0.01
28	7.8 ± 1.0	0.3 ± 1.1	0.01	19.7 ± 1.2	16.1 ± 1.4	0.10
35	8.7 ± 1.1	0.8 ± 1.1	0.01	25.7 ± 1.2	20.8 ± 1.5	0.02
42	10.3 ± 1.4	−0.3 ± 1.6	0.01	28.8 ± 1.7	23.0 ± 1.9	0.05
Survival	22/30	19/33		31/31	31/31	
		Ad libitum feeding				
7	9.3 ± 1.6	4.1 ± 1.3	0.05	36.0 ± 1.8	27.7 ± 2.1	0.2
14	9.8 ± 2.1	4.9 ± 1.9	0.1	68.0 ± 2.5	61.3 ± 3.4	0.2
21	9.4 ± 2.5	3.3 ± 1.9	0.1	101.4 ± 4.6	95.0 ± 4.8	0.4
28	8.7 ± 2.3	2.9 ± 1.9	0.1	145.9 ± 5.7	127.2 ± 7.5	0.1
35	7.8 ± 2.2	−1.8 ± 1.7	0.01	179.0 ± 9.7	153.0 ± 10.3	0.1
Survival	12/14	13/14		14/14	14/14	

[1] Osborne-Mendel strain.
[2] Mean ± SE of mean.

the rats of each sex were also fed thalidomide, After 7 weeks, riboflavin was added to the diets of all groups and the experiment was continued for another 9 weeks. Again thalidomide retarded the growth of both sexes during the depletion period (table 4). During the repletion period, thalidomide did not exert a significant effect on the growth of the rats. Thus, these rats served as their own controls, further establishing the fact that riboflavin depletion, under the conditions described, mark-

TABLE 3

Growth-depressing effect of thalidomide in female rats as affected by riboflavin depletion [1]

Riboflavin, mg/kg diet	0	0		6.2	6.2	
Thalidomide, g/kg diet	0	7.5		0	7.5	
				Cumulative weight gain		
days	*g*	*g*	*P value*	*g*	*g*	*P value*
			Osborne-Mendel strain			
7	1.0 ± 07 [2]	−2.2 ± 1.2	0.05	17.2 ± 1.0	8.0 ± 4.1	0.1
19	4.4 ± 1.8	4.0 ± 2.2	0.5	55.6 ± 2.4	44.0 ± 2.1	0.01
29	9.4 ± 0.5	8.2 ± 4.1	0.5	83.6 ± 2.4	79.8 ± 2.8	0.3
39	11.2 ± 2.0	4.8 ± 3.5	0.2	108.4 ± 4.1	108.4 ± 5.8	0.5
46	15.0 ± 1.9	1.6 ± 2.9	0.01	123.8 ± 5.1	121.4 ± 6.6	0.5
Survival	5/5	5/5		5/5	5/5	
			Sprague-Dawley strain			
7	14.6 ± 1.0	13.2 ± 0.8	0.2	29.3 ± 2.0	19.8 ± 2.0	0.02
14	19.5 ± 1.9	15.3 ± 2.1	0.2	58.1 ± 2.5	51.5 ± 2.6	0.2
21	29.8 ± 3.1	16.3 ± 2.8	0.01	90.1 ± 2.8	81.3 ± 3.8	0.1
28	38.1 ± 2.7	19.0 ± 3.6	0.01	113.1 ± 4.3	110.0 ± 3.0	0.5
35	43.0 ± 2.8	19.0 ± 3.8	0.01	135.9 ± 2.4	137.6 ± 2.3	0.5
42	46.8 ± 2.9	16.5 ± 3.8	0.01	171.1 ± 5.5	163.1 ± 1.5	0.2
49	50.6 ± 5.1	15.5 ± 3.9	0.01	186.1 ± 6.4	175.0 ± 2.9	0.2
Survival	9/10	10/10		5/6	6/6	

[1] Ad libitum feeding.
[2] Mean ± SE of mean.

TABLE 4

Effect of riboflavin depletion followed by riboflavin repletion on growth-depressing effect of thalidomide

Dietary thalidomide, g/kg	0	7.5		0	7.5	
				Cumulative weight gain		
days	*g*	*g*	*P value*	*g*	*g*	*P value*
	Female rats [1]			Male rats [1]		
			Depletion period [2]			
14	20.4 ± 4.5	19.2 ± 2.4	0.5	12.2 ± 1.1	15.0 ± 2.2	0.1
21	30.4 ± 3.6	18.0 ± 2.3	0.02	15.8 ± 2.1	12.4 ± 2.9	0.4
28	37.4 ± 4.0	18.4 ± 5.1	0.02	25.2 ± 3.6	13.8 ± 3.0	0.05
35	42.8 ± 6.4	17.6 ± 5.6	0.02	29.8 ± 4.8	12.6 ± 4.0	0.05
42	44.2 ± 5.4	16.6 ± 6.0	0.01	33.6 ± 6.4	9.2 ± 4.1	0.02
49	45.4 ± 3.9	17.2 ± 6.4	0.01	37.6 ± 7.6	8.4 ± 4.5	0.02
			Repletion period [3]			
14	94.8 ± 7.8	77.3 ± 4.9	0.1	115.0 ± 9.4	92.7 ± 7.0	0.2
21	121.6 ± 7.0	97.8 ± 3.2	0.02	154.8 ± 16.5	134.7 ± 5.9	0.4
28	145.2 ± 6.4	121.6 ± 5.9	0.05	203.2 ± 17.0	180.3 ± 7.2	0.3
35	155.8 ± 7.3	135.0 ± 7.6	0.1	231.2 ± 17.5	212.3 ± 8.4	0.5
42	169.8 ± 8.2	152.8 ± 0.2	0.3	253.8 ± 17.0	234.0 ± 4.9	0.4
49	180.6 ± 11.1	163 ± 9.8	0.3	288.0 ± 17.8	268.0 ± 12.5	0.4
63	196.0 ± 11.2	183.0 ± 13.2	0.5	327.6 ± 19.5	308.3 ± 11.5	0.4
Survival	5/5	4/5		5/5	4/5	

[1] Sprague-Dawley strain.
[2] Average cumulative weight gain ± SE of mean during period in which rats were fed diet lacking in riboflavin.
[3] Average cumulative weight gain ± SE of mean starting from day 49 when diet was supplemented with 6.2 mg riboflavin/kg.

edly enhanced the growth-depressing properties of thalidomide.

Thalidomide lowered the white cell count in the riboflavin-deficient rats but had no such effect in rats receiving riboflavin (table 5). Thalidomide had no effect on the red blood cell count or hemoglobin concentration (not shown). Thus, the toxic response to thalidomide has again been strikingly accentuated by the riboflavin deficiency.

To determine whether thalidomide was actually interfering with the metabolism of flavins or their role as coenzymes, the livers of the male rats described in the ad libitum and equalized feeding studies were analyzed for the FAD-dependent enzymes DAO and SDH; the flavin coenzymes, FMN and FAD, were also determined (table 6). The activity of SDH was somewhat depressed by thalidomide in both experiments but the effect was statistically significant only in the riboflavin-deficient rats of the equalized feeding group. In another experiment (table 7) the succinoxidase of livers from riboflavin-deficient rats, but not control rats, was significantly reduced by thalidomide fed for 7 weeks. Since thalidomide failed to depress liver DAO or the synthesis of liver flavin coenzymes it is unlikely that the drug acts as a riboflavin antagonist. Burch et al. (18) have shown liver DAO to be very sensitive to riboflavin depletion, and Miller et al. (19) have demonstrated a marked reduction of FAD in the livers of rat embryos from mothers fed galactoflavin, a known flavin antagonist. Galactoflavin also very markedly reduces the DAO activity of growing rats.[7] In some of the cases, livers from thalidomide-treated rats had a somewhat higher DAO activity than that of the corresponding controls. Also thalidomide apparently doubled the liver succinoxidase of control rats (table 7). These results are of interest in view of the observation of Midtvedt (20) that thalidomide reduced the lag phase of the growth of riboflavin-requiring organism, *Lactobacillus delbrueckii*. The lowering of succinic dehydrogenase and succinoxidase in the livers of riboflavin-depleted rats treated with thalidomide may be the result of a nonspecific response to stress, since these enzyme systems have been reported to be responsive to traumatic situations such as starvation (21) and vigorous exercise (22).

There was no significant evidence of gross or histological pathology in the livers of rats fed thalidomide with or without riboflavin supplementation. No gross changes were noted in the other organs of the rats treated with thalidomide. This correlates well with the absence of any

[7] See footnote 3.

TABLE 5

Effect of thalidomide and riboflavin-deficiency on blood cell counts of rats after 7 weeks on treatment

Dietary supplements		No. rats/ group	White cells	Red cells
Riboflavin	Thalidomide			
mg/kg	*g/kg*		*1000/mm³*	*million/mm³*
		Osborne-Mendel females		
6.2	0	5	10.75 ± 0.62 [1]	7.92 ± 0.20
6.2	7.5	5	12.04 ± 2.10	7.15 ± 0.29
0	0	4	11.39 ± 0.77	7.26 ± 0.57
0	7.5	5	4.57 ± 1.38	6.98 ± 0.26
		Sprague-Dawley females		
6.2	0	5	16.22 ± 1.61	7.29 ± 0.28
6.2	7.5	5	15.32 ± 1.00	8.31 ± 0.10
0	0	5	17.04 ± 0.49	9.11 ± 0.59
0	7.5	5	8.65 ± 0.90	8.53 ± 0.23
		Sprague-Dawley males		
0	0	5	17.18 ± 3.42	8.02 ± 0.22
0	7.5	5	9.18 ± 2.08	8.39 ± 0.35

[1] Mean ± SE of mean.

TABLE 6

Effect of thalidomide and riboflavin deficiency on liver flavin enzymes and coenzymes of rats [1]

Dietary supplements		SDH [2]	GDH	DAO	FAD	FMN	Total riboflavin
Riboflavin	Thalidomide						
mg/kg	*g/kg*	$\Delta OD_{600}/hr/g$		*μmoles/hr/g*	*μg/g*	*μg/g*	*μg/g*
				Equalized feeding (4 g/rat/day) [3]			
6.2	0	175 ± 18(21)	344 ± 29(17)	76.4 ± 6.7(14)	22.8 ± 1.1(25)	10.5 ± 0.7(25)	33.3 ± 0.8(25)
6.2	7.5	165 ± 8 (23)	313 ± 27(18)	92.4 ± 8.2(14)	25.3 ± 1.1(24)	10.4 ± 0.6(24)	35.7 ± 0.9(24)
0	0	143 ± 16(19)	276 ± 25(16)	36.9 ± 5.2(13)	8.5 ± 0.6(22)	2.2 ± 0.4(22)	10.7 ± 0.8(22)
0	7.5	98 ± 8 (17)	297 ± 46(13)	40.4 ± 5.1(11)	9.1 ± 0.7(19)	2.5 ± 0.4(19)	11.6 ± 1.3(19)
				Ad libitum feeding [4]			
6.2	0	116 ± 19(10)	—	99.4 ± 7.1(12)	20.2 ± 1.5(9)	8.8 ± 0.8(9)	29.0 ± 1.5(9)
6.2	7.5	94 ± 8 (9)	—	102.8 ± 5.6(12)	22.3 ± 0.7(9)	8.8 ± 0.5(9)	31.1 ± 0.9(9)
0	0	87 ± 12(9)	—	34.3 ± 4.2(11)	6.8 ± 0.2(9)	2.5 ± 0.3(9)	9.3 ± 0.3(9)
0	7.5	61 ± 14(9)	—	44.0 ± 3.5(12)	6.6 ± 0.4(10)	2.6 ± 0.8(10)	9.2 ± 0.5(10)

[1] Osborne-Mendel rats used. All values represent mean ± SE of mean. Figures in parentheses denote number of rats per group.
[2] SDH = succinic dehydrogenase, GDH = glutamic dehydrogenase, DAO = D-amino oxidase, FAD = flavin adenine dinucleotide, FMN = flavin mononucleotide plus free riboflavin.
[3] Livers analyzed after 42 days of feeding experimental diets.
[4] Livers analyzed after 35 days of feeding experimental diets.

TABLE 7

Effect of thalidomide and riboflavin deficiency on liver succinoxidase of 7-week-old female rats [1]

Dietary supplements		Enzyme activity	
Riboflavin	Thalidomide		
mg/kg	*g/kg*	*μl O_2/hr/mg*	*P value*
6.2	0	2.41 ± 0.21 [2]	
6.2	7.5	4.55 ± 0.37	0.01
0	0	2.54 ± 0.43	
0	7.5	1.51 ± 0.13	0.05

[1] Osborne-Mendel strain.
[2] Mean of 6 animals per group ± SE of mean.

appreciable increase in the activities of plasma lactic acid dehydrogenase, alkaline phosphatase or transaminase. Analyses of liver protein, liver moisture, liver total lipid or liver weight indicated a small increase in the liver-body weight ratios of rats fed thalidomide, with the increase slightly more apparent in the riboflavin-supplemented group (table 8). Enlarged livers have been shown to be a consequence of ingestion of other drugs such as the barbituates (23) in which case the enlargement has been attributed to enhanced cell proliferation.

Folic acid studies. Since thalidomide has been suggested as a folic acid antagonist (6), growth experiments similar to those performed with riboflavin-deficient diets were repeated with diets deficient and sufficient in folic acid. Under the conditions of these experiments, thalidomide, at the same levels used in the riboflavin experiments (0.75%), exerted a significant effect on the growth of the rats both in the folic acid-deficient and control groups (table 9). However, these results may have been modified by the sulfa drug which was included in the diet to promote a folic acid deficiency. The growth depression induced by thalidomide was not enhanced by the folic acid-deficient state as it was by the riboflavin-deficient state. That a true folic acid deficiency was induced in the rats was evidenced both by the weight (table 9) and blood cell reduction (table 10) of rats fed the folic acid-deficient diet relative to the rats fed the control. With these diets thalidomide induced a white cell reduction in both groups of rats, whereas with the riboflavin test diets, only the riboflavin-deficient rat responded to the leucopenia-producing effect of the drug. The sulfa drug, or some other factor

TABLE 8

Effect of thalidomide and riboflavin deficiency on liver composition of rats [1]

Dietary supplements		Liver weight	Protein	Moisture	Fat
Riboflavin	Thalidomide				
mg/kg	*g/kg*	*% body wt*	*%*	*%*	*%*
		Equalized feeding (4 g/rat/day) [2]			
6.2	0	3.75 ± 0.09 [3] (30) [4]	19.9 ± 12.1 (28)	71.3 ± 0.8 (10)	—
6.2	7.5	4.45 ± 0.08 (32)	20.8 ± 3.6 (29)	71.4 ± 0.4 (10)	—
0	0	5.96 ± 0.61 (26)	19.9 ± 1.7 (26)	71.8 ± 0.7 (8)	—
0	7.5	6.80 ± 0.33 (21)	19.4 ± 5.4 (20)	74.0 ± 1.2 (5)	—
		Ad libitum feeding [5]			
6.2	0	4.05 ± 0.17 (12)	23.0 ± 6.3 (12)	74.4 ± 0.4 (12)	4.38 ± 0.56 (5)
6.2	7.5	4.46 ± 0.17 (12)	22.3 ± 6.3 (12)	72.6 ± 0.2 (12)	4.26 ± 0.21 (5)
0	0	5.90 ± 0.26 (11)	20.1 ± 6.3 (11)	73.9 ± 0.6 (11)	3.91 ± 0.77 (6)
0	7.5	6.26 ± 0.22 (12)	19.0 ± 6.4 (12)	72.1 ± 2.2 (12)	3.68 ± 0.47 (6)

[1] Osborne-Mendel male rats used.
[2] Liver of rats analyzed after 42 days of feeding experimental diet.
[3] Mean ± SE of mean.
[4] Figures in parentheses denote number of rats per group.
[5] Livers of rats analyzed after 35 days of feeding experimental diet.

TABLE 9

Growth-depressing effect of thalidomide as affected by folic acid depletion [1]

Folic acid, mg/kg	0	0		2.0	2.0	
Thalidomide, g/kg	0	7.5		0	7.5	
		Cumulative weight gain				
days	*g*	*g*	*P value*	*g*	*g*	*P value*
14	56.9 ± 3.4 [2]	39.7 ± 2.9	0.01	55.9 ± 3.8	42.3 ± 3.7	0.05
21	61.7 ± 2.4	46.8 ± 2.1	0.01	81.6 ± 5.4	55.7 ± 4.3	0.01
28	111.0 ± 3.6	75.1 ± 3.6	0.01	137.8 ± 8.8	83.6 ± 7.0	0.01
35	141.8 ± 4.1	94.1 ± 2.6	0.01	183.1 ± 9.0	115.0 ± 10.7	0.01
42	167.8 ± 5.9	114.4 ± 5.2	0.01	243.4 ± 8.9	159.1 ± 8.7	0.01
49	195.4 ± 11.9	138.2 ± 6.2	0.01	281.0 ± 10.5	188.1 ± 10.3	0.01
56	195.4 ± 11.9	138.2 ± 7.2	0.01	315.8 ± 10.0	212.0 ± 12.4	0.01
Survival	9/10	9/10		9/9	9/9	

[1] Sprague-Dawley female rats used.
[2] Mean ± SE of mean.

TABLE 10

Effect of thalidomide and folic acid deficiency on blood cell counts of 8-week-old rats [1]

Folic acid	Thalidomide	White cells	Red cells
mg/kg	*g/kg*	*1000/mm³*	*million/mm³*
2.0	0	12.97 ± 0.85 [2] (9) [3]	8.27 ± 0.47 (5)
2.0	7.5	9.81 ± 0.56 (7)	6.83 ± 0.28 (5)
0	0	5.69 ± 0.41 (9)	5.97 ± 0.46 (4)
0	7.5	4.37 ± 0.45 (9)	6.43 ± 0.52 (4)

[1] Sprague-Dawley strains.
[2] Mean ± SE of mean.
[3] Figures in parentheses denote number of animals in experimental group.

in the folic acid test diet, may have synergistically enhanced the toxicity of thalidomide, since a centrolobular necrosis was observed in the livers of the 5 rats examined. Special staining techniques revealed swollen fat-filled cells surrounding many of the central veins in these livers. Only the livers of rats receiving thalidomide and folic acid and the corresponding controls were histologically examined, since no gross lesions were apparent in the livers of folic acid-deficient rats receiving thalidomide. Chemical analysis of the livers showed no change in protein or nucleic

acid content but a slight increase in total lipid content (6.14% vs. 5.12%) relative to that of the livers of the control rats. A single experiment was made in which the urine of control rats and those receiving dietary thalidomide for 8 weeks was analyzed for formiminoglutamic acid; results indicated no increase in the concentration of this compound in the urine of the latter group. This observation plus other evidence present in this report including the failure of thalidomide to induce a consistent anemia (tables 5 and 10) tend not to implicate thalidomide as a folic acid antagonist (24).

The results of these studies do not elucidate the mode of action of thalidomide as a teratogenic agent. It is realized that the biochemistry of the developing embryo may have unique features which make it sensitive to chemical and physical factors normally innocuous to a growing or mature animal. However, by subjecting a growing animal to dietary or other stresses it may be possible to simulate some of the conditions in the rapidly growing and demanding embryo. In any event, if such a stress renders an otherwise relatively innocuous drug toxic, then we may have a means not only to gain insight into the mechanism of action of the toxicity of this drug in the embryo but also possibly to obtain an improved understanding of the role of an essential nutrient in the body. Perhaps dietary stress may also facilitate the early detection of a mildly and insidously toxic compound which otherwise may be overlooked. Thus this work is apparently the first report of a toxic response to ingested thalidomide by an adult rat, even though the drug has been implicated in the development of specific types of neuropathies in the human adult (25).

In conclusion, it may be stated that although the production of a riboflavin deficiency in rats did promote the apparent toxicity of thalidomide, the role of this dietary stress in this phenomenon cannot be explained yet.

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