Further Studies of the Effect of Linoleic Acid on Reproduction in the Hen

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ABSTRACT Single Comb White Leghorn female pullets were reared from hatching to 25 weeks of age with a low fat purified diet calculated to contain 0.005% linoleic acid (18:2). The pullets were then distributed into 6 groups of 20 birds each and given safflower oil at increments calculated to furnish zero, 10, 20, 40, 80, and 250 mg of 18:2/bird/day respectively. An increase in egg production, egg size, fertility and hatchability paralleled an increase in dietary 18:2 with the exception that hatchability still remained zero at the 10-mg level. Only 20 mg of 18:2 daily were necessary in this extracted, purified diet to increase fertility from 73.4 to 92.5%, and to decrease early embryonic mortality from 31.0 to 10.8%. Higher levels of 18:2 had no further effect on fertility or early embryonic mortality. The presence of quantities of eicostrienoic acid (20:3) in the plasma and yolk lipids from essential fatty acid-deficient hens suggests a possible relationship between the development or expression of the various reproductive characteristics and the tissue level of 20:3. The levels of the 20:3 acid in the tissues decreased with each increase in dietary 18:2. These changes were reflected by increases in egg size, egg production, hatchability, and decreased early embryonic mortality.

Major considerations in the study of the essential fatty acids (EFA) are the EFA status of the experimental animal at the start of the experiment, and the length of the experiment. This was demonstrated by Miller et al. (1) who reported that pullets reared for 16 weeks with a practical-type diet and then maintained with a low fat diet for 40 weeks still retained substantial amounts of linoleic (18:2) and arachidonic (20:4) acids in the fat of the heart, thigh, and breast muscle. Later, Menge et al. (2) reported that 20-week old pullets reared with an 18:2-deficient diet from hatching had no detectable amounts of 18:2 or 20:4 in the heart, thigh, and breast muscle fat. Eicosatrienoic acid, tentatively identified as the 5,8,11 isomer (20:3), in the tissues of these pullets was 4 to 10% higher than that in the tissues of the pullets as reported by Miller et al. (1). The severity of the deficiency was further demonstrated by increased susceptibility to respiratory infections, poor egg production, small egg size, low fertility, and zero hatchability. Zero hatchability represents the most acute deficiency of 18:2 that has been reported to date. The study reported here was designed to investigate further the effect of 18:2 on egg production, egg size, fertility, and hatchability.

EXPERIMENTAL PROCEDURE

Two hundred Single Comb White Leghorn pullets (commercial strain) were reared and maintained with an 18:2-deficient grower and layer diet (table 1) containing approximately 0.005% of 18:2 as determined by acid hydrolysis (3) and gas-liquid chromatography (GLC). Hydrogenated coconut oil [1] was added to these diets to facilitate absorption of fat-soluble vitamins and also for its effect on the depletion of EFA. All diets were isocaloric and isonitrogenous. Cellulose and carbohydrate were adjusted to compensate for oil supplementations. The diets were pelleted by the method of McWard and Scott (4). The combs and wattles of the one-day-old chicks were removed to eliminate the difficulty experienced with the large combs and wattles of hens in laying cages.

The pullets began to exhibit symptoms of bronchitis at about 14 weeks of age, and until they were approximately 32 weeks of age they suffered recurrent mortality attributed primarily to a respiratory syndrome similar to that described by Ross and Adamson (5), and Hopkins et al. (6). Chlortetracycline (1 g/kg) and nystatin

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[1] Supplied by the Procter and Gamble Company, Cincinnati.

TABLE 1

Composition of diets

	Grower diet	Layer diet
	%	%
Isolated soy protein [1]	18.0	18.0
Casein [1]	5.0	5.0
Powdered cellulose [1,2]	4.0	10.0
Gelatin	5.0	5.0
Coconut oil (hydrogenated)	2.0	2.5
Mineral mix	6.0 [3]	12.09 [4]
Vitamin mix	0.5 [5]	0.5 [6]
Methionine hydroxy analogue (90%)	0.56	0.56
Choline chloride (70%)	0.35	0.35
Antioxidant [7]	0.015	0.015
Glucose monohydrate	58.575	45.985

[1] The soy protein, casein, and powdered cellulose were extracted with 95% MeOH.

[2] Solka Floc, Brown Company, Berlin, New Hampshire.

[3] Mineral mix supplied the following: (in %) limestone flour, 0.8; dicalcium phosphate (Ca, 25%; P, 18.5%), 3.2; KH_2PO_4, 1.0; NaCl, 0.3; $MgSO_4$, 0.1; $FeC_6H_5O_7 \cdot 5H_2O$, 0.06; $MnSO_4 \cdot H_2O$, 0.03; $Cu(CH_3COO)_2 \cdot H_2O$, 0.003; KI, 0.004; $ZnCO_3$, 0.01; H_3BO_3, 0.001; $CoSO_4 \cdot 7H_2O$, 0.0002; $Na_2SeO_4 \cdot 10H_2O$, 0.00005; $Na_2MoO_4 \cdot 2H_2O$, 0.0009; extracted powdered cellulose to 6% of diet.

[4] Mineral mix supplied the following: (in %) limestone flour, 8.0; dicalcium phosphate (Ca, 25%; P, 18.5%), 2.5; KH_2PO_4, 1.0; NaCl, 0.3; $MgCO_3$, 0.18; $FeC_6H_5O_7 \cdot 5H_2O$, 0.06; $MnSO_4 \cdot H_2O$, 0.03; $ZnCO_3$,0.0125; $Cu(CH_3COO)_2 \cdot H_2O$, 0.004; $Na_2SeO_4 \cdot 10H_2O$, 0.00005; $Na_2MoO_4 \cdot 2H_2O$, 0.0006; H_3BO_3, 0.001; $CoSO_4 \cdot 7H_2O$, 0.0002; and KI, 0.004.

[5] Vitamin mix supplied the following: (mg/kg of diet) thiamine·HCl, 100; riboflavin, 16; Ca D-pantothenate, 25; pyridoxine·HCl, 10; folacin, 4; menadione, 5; niacin, 100; biotin, 0.6; vitamin B_{12} (0.1%), 20; vitamin A (500,000USP/g), 36; vitamin D_3 (200,000 ICU/g), 7; *d*-α-tocopheryl acetate (1360 IU/g), 26; extracted powdered cellulose to 0.5% of diet.

[6] Vitamin premix supplied the following: (mg/kg of diet) thiamine·HCl, 125; riboflavin, 20; Ca D-pantothenate, 31.25; pyridoxine·HCl, 12.5; folacin, 5; menadione, 6.25; niacin, 125; biotin, 0.75; vitamin B_{12} (0.1%), 18.75; inositol, 125; vitamin A (500,000 USP/g), 45; vitamin D_3 (200,000 ICU/g), 10; *d*-α-tocopheryl acetate (1360 IU/g), 50; powdered cellulose (extracted) to 0.5% of diet.

[7] 1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline. Monsanto Chemical Company, St. Louis.

(200 mg/kg) were used in an attempt to reduce the losses. At 20 weeks of age the pullets were placed into individual cage laying batteries in a constant-temperature room (21°) and fed the 18:2-deficient layer ration (table 1).

At 25 weeks of age the pullets were divided at random into 6 groups of 20 birds each and fed the experimental diets. Calculated on the basis of daily feed consumption, groups 1 through 6 received zero, 10, 20, 40, 80, and 250 mg of 18:2/hen/day, respectively. Safflower oil, containing 77.7% of 18:2 by GLC analysis, was used as the source of 18:2. It was assumed that any responses would be due to 18:2 only, since Menge et al. (2) had reported that 18:2 (95%) which they prepared from safflower oil was just as effective as safflower oil in stimulating increased egg production, egg size, fertility and hatchability.

The pullets were artificially inseminated twice each week with 0.05 ml pooled Single Comb White Leghorn semen. Individual body weights were recorded monthly and egg production was tabulated daily. Before the eggs were set they were weighed, and after 6 days of incubation, they were candled to determine fertility and early embryonic mortality. Fatty acid analyses were made on eggs collected at the start of the experiment, after 8 weeks, and again after 20 weeks. Blood samples were also taken at the start of the experiment and again after 20 weeks for analyses. Methods for determination of plasma and yolk fatty acids were described in a previous paper (1).

RESULTS

The effect of increasing levels of 18:2 on egg production, egg weight, fertility, early embryonic mortality, and hatchability for a 5-month experimental period is shown in table 2. With the exception of the 40-mg level (group 4), increasing levels of the fatty acid stimulated a concomitant increase in average egg production. The production data for group 4 are not biologically sound and there is ample reason to suspect that egg production from this group was suppressed in some way other than by treatment or position effect, or both.

A general increase in egg weight was observed with each increase in dietary 18:2. The 10-mg level of 18:2 which stimulated a pronounced effect on egg production, also promoted the most substantial increase in egg size.

Dietary 18:2 had a striking effect on the percentage of fertile eggs produced, and also on early embryonic mortality (6 days). Twenty milligrams 18:2/hen/day (group 3) were apparently sufficient for 92.5% fertility and the reduction of early mortality from 31.0 to 10.8%. Higher levels of 18:2 did not stimulate a further increase in fertility or have a further effect on early embryonic mortality.

TABLE 2

Effect of linoleic acid on reproduction in the hen through a 5-month experimental period [1]

Group	Linoleic acid fed [2]	Average				
		Egg production	Egg wt	Fertility	Early mortality	Hatchability
	mg	%	*g*	%	%	%
1	0	39.4 [a]	40.4 [a]	73.4 [a]	31.0 [a]	0
2	10	56.5 [c]	43.3 [b]	79.8 [a]	22.5 [b]	0
3	20	58.7 [c]	43.4 [b]	92.5 [b]	10.8 [c]	1.4 [a]
4	40	50.9 [b]	43.6 [b]	90.6 [b]	10.0 [c]	10.3 [b]
5	80	63.9 [d]	44.9 [c]	90.6 [b]	9.2 [c]	15.3 [c]
6	250	69.3 [e]	46.3 [d]	89.1 [b]	8.5 [c]	51.6 [d]

[1] Means with different superscripts are significantly different at the 1% level according to Duncan's multiple range test (19).
[2] Supplied by safflower oil.

Groups 1 and 2 receiving zero and 10 mg 18:2/hen/day, respectively, produced eggs with zero hatchability. No chicks hatched unless the hens received at least 20 mg of 18:2/day. Increased quantities of dietary 18:2 resulted in concomitant increases in hatchability.

Fatty acid analyses of yolks obtained at the start of the experiment, after 8 weeks, and again at the end of 20 weeks are presented in table 3. After 8 weeks of supplementation with graded levels of 18:2, the percentage of oleic acid (18:1) and 20:3 decreased with each increase in dietary 18:2 with the exception of 18:1 in group 6 and 20:3 in group 3. The percentage of 18:2 in yolk fat did not show a concurrent increase. In fact, 18:2 was not detected in the yolk fat of eggs from hens receiving 10 mg or less of 18:2/day. The analyses of fatty acids in yolk fat of eggs from hens fed the experimental diets for 20 weeks was similar to that observed at 8 weeks with a few exceptions. Traces of 18:2 and 20:4 were noted at the 10- and 20-mg level of dietary 18:2, and also traces of 20:4 at the 40-mg level at 20 weeks, but not at 8 weeks. At 20 weeks there also was a gradual decrease in 20:3 and an increase in 20:4 in the yolk fat of eggs from the groups receiving 18:2 at increasing levels.

Table 4 presents the fatty acid analysis of plasma taken after 20 weeks of experimentation. No significant changes in the percentage of palmitoleic acid (16:1) or 18:1 were noted in the groups receiving zero to 250 mg of 18:2 daily. Linoleic acid was not detected in the plasma fat of hens receiving less than 40 mg of 18:2. Arachidonic acid was not present in the plasma of hens receiving less than 20 mg of 18:2, and only in trace quantities at this level. Plasma 20:3, which was pres-

TABLE 3

Fatty acid composition of egg yolk lipid

Group	Linoleic acid fed [1]	Fatty acid [2,3]													
		16:1	18:0	18:1	18:2	18:3	20:3 [4]	20:4	16:1	18:0	18:1	18:2	18:3	20:3 [4]	20:4
		8 Weeks [5]							20 Weeks [5]						
	mg	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	0	5.8	6.4	61.7	0.0	0.8	2.0	0.0	6.6	5.6	59.9	0.0	0.7	1.7	0.0
2	10	6.9	5.7	60.1	0.0	0.6	1.4	0.0	6.4	6.4	59.8	0.2	0.8	1.5	0.1
3	20	7.2	5.6	58.7	0.1	0.7	1.4	0.0	7.3	5.8	58.3	0.2	0.6	1.2	0.2
4	40	6.8	6.6	57.3	0.4	0.6	1.0	0.0	7.3	5.0	59.0	0.2	0.6	1.0	0.3
5	80	7.9	7.3	49.1	0.3	0.4	0.9	0.2	7.7	6.3	54.6	1.0	0.6	0.9	0.5
6	250	6.6	7.5	52.0	1.7	0.5	0.6	0.5	7.1	6.0	54.3	2.6	0.3	0.4	0.7

[1] Linoleic acid supplied by safflower oil.
[2] The first figure represents the number of carbon atoms; the second, the number of double bonds.
[3] Yolk fatty acids prior to start of experiment: (in %) 16:1, 6.2; 18:0, 6.8; 18:1, 59.7; 18:2, 0.0; 18:3, 0.8; 20:3, 2.3; 20:4, 0.0.
[4] The 20:3 acid is tentatively identified as the 5,8,11 isomer of eicosatrienoic acid.
[5] Yolk fatty acids after 8 and 20 weeks of experimentation.

TABLE 4

Fatty acid composition of plasma lipids

Group	Linoleic acid fed [1]	Fatty acid [2,3]						
		16:1	18:0	18:1	18:2	18:3	20:3 [4]	20:4
		20 Weeks [5]						
	mg	%	%	%	%	%	%	%
1	0	5.3	8.5	57.9	0.0	0.9	2.6	0.0
2	10	7.2	7.6	57.7	0.0	0.7	1.4	0.0
3	20	8.6	6.2	58.2	0.0	0.5	1.6	0.1
4	40	7.3	7.7	57.8	0.8	0.7	1.4	0.2
5	80	8.8	7.8	48.6	4.0	1.2	1.0	0.2
6	250	7.1	6.8	52.7	2.2	0.4	0.4	1.1

[1] Linoleic acid supplied by safflower oil.
[2] The first figure represents the number of carbon atoms; the second, the number of double bonds.
[3] Plasma fatty acids from pullets prior to start of experiment: (in %) 16:1, 5.9; 18.0, 7.8; 18:1. 59.0; 18:2, 0.0; 18:3, 0.6; 20:3, 3.5; 20:4, 0.0.
[4] The 20:3 acid is tentatively identified as the 5,8,11 isomer of eicosatrienoic acid.
[5] Fatty acid analyses of plasma lipids after 20 weeks' experimentation.

ent in all groups, decreased with each increase in supplemental 18:2 with the exception of the 20- and 40-mg level of 18:2.

DISCUSSION

Several workers have stated that dietary 18:2 had no effect on rate of production in the EFA-deficient hen (7, 8). In contradiction, reports from other laboratories (2, 9, 10) show that the administration of a dietary source of 18:2 results in increased production from EFA-deficient hens. The data presented in table 2 leave little doubt as to the efficacy of 18:2 in increasing egg production from hens severely depleted of EFA.

Egg weights obtained from the EFA-deficient hen are dependent upon the depletion of the bird and the 18:2 content of the diet. Increasing the levels of 18:2 had a positive effect on increasing egg size (table 2). March and Biely (11) reported that amino acid balance and not protein level was the factor limiting egg size, whereas Fisher and Shapiro (12) stated that egg size was influenced by increased protein intake. On the other hand, Jensen et al. (13) showed that crude corn oil was effective in increasing egg size. Later Shutze and Jensen (14) obtained results to show that 18:2 was responsible for the major part of the increase in egg size noted when highly unsaturated vegetable oils were fed to hens. An increase in egg size attributable to dietary oil was demonstrated by other workers using either a practical (15, 16) or a purified diet (9, 17). Recently, Blamberg et al. (18) reported that metabolizable energy had a greater effect on egg weight than did 18:2 intake. The diets used by these workers contained 1.4% of 18:2. The above reports are not necessarily in conflict with our data for it may well be that after the requirement for 18:2 has been met, amino acid balance, protein intake, or metabolizable energy are the next limiting factors in increasing egg size.

The data on the average percentage of fertile eggs and early embryonic mortality for the 20-week experimental period show the pronounced effect (table 2) of small amounts of 18:2 on fertility and early mortality. The percentage of fertile eggs from all 18:2-supplemented groups ranged from 91 to 100% for the last hatch of this study in comparison with 79% for group 1 (zero milligrams of 18:2). The percentage of early mortality for this same hatch ranged from 4 to 12% in comparison with 20% for group 1.

The most sensitive indication of an EFA-deficient hen, according to our data (table 2), is the observation of zero hatchability. These data confirm a previous study (2) and demonstrate the need of the developing embryo for 18:2. Jensen et al. (13) presented evidence for the existence of an unidentified factor in corn oil that is necessary for hatchability. Marion and Edwards (9) reported that the addition of corn oil to a low fat diet fed to fat-deficient hens stimulated a significant increase in hatchability. Later Jensen and Shutze (8) re-

ported a depression in hatchability of eggs laid by EFA-deficient hens. These data agree in part with those obtained in our laboratory. The production of zero hatchability can be accomplished only by long-term feeding of a diet practically devoid of 18:2. The hatching of chicks from "EFA-deficient" dams is a definite indication of the presence of small quantities of 18:2 in the feed and consequently in the tissues of the birds where it is metabolized or utilized to support reproductive functions.

Fatty acid analyses of plasma and yolk fat (tables 3 and 4) show that the pullets were depleted of their stores of 18:2 prior to the start of the experiment. Group 1 (zero milligrams of 18:2) maintained this stage of depletion throughout the 20 weeks of the study. With few exceptions the levels of the 20:3 acid in the tissues decreased with each increase in dietary 18:2. These changes were reflected by increases in egg size, egg production, fertility, and hatchability, and decreased early embryonic mortality. None of the other fatty acids exhibited a similar relationship except the 20:4 acid in the yolk fat and then only after 20 weeks. The 20:3 acid, however, began to react immediately to increased levels of dietary 18:2 in both the plasma and the yolk fat. These data show that the 20:3 acid is most sensitive to dietary 18:2 and indicate that the amount of 20:3 in the tissues of the EFA-deficient bird bears a definite relationship to the expression of the several reproductive functions of the chicken. This is in agreement with Marion and Edwards (17) who have stated that the level of 20:3 in the liver and heart tissues is negatively associated with responses in egg size, production, and hatchability when dietary sources of 18:2 are supplied.

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Absorption of Cu^{64}, Zn^{65}, Mo^{99}, and Fe^{59} from Ligated Segments of the Rat Gastrointestinal Tract

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ABSTRACT Portions of rat intestinal tract were ligated to provide the following isolated, in vivo, segments: stomach: duodenum, mid-section, or ileum. In successive studies, Cu^{64}, Zn^{65}, Fe^{59}, or Mo^{99} was injected into one of the ligated segments. After a predetermined period of time, the rats were killed and tissue samples were taken for isotope analysis. On the basis of these tissue counts, the following relative rates of absorption were indicated. For Cu^{64}, absorption was greatest from the stomach and declined as the isotope was placed further away from the pylorus. Zn^{65} and Fe^{59} were taken up most rapidly from the duodenum, somewhat more slowly from the ileum and the mid-section, and with the least absorption occurring from the stomach. Mo^{99} was absorbed readily from the stomach and all 3 intestinal segments. Absorption from the 3 intestinal segments was at similar rates and all three yielded significantly higher rates than the stomach.

Little is known of either the site or the mechanism for the absorption of copper from the intestine. However, a number of other ions, both chemically similar and chemically dissimilar, have been shown to affect the metabolism of copper. Of these, zinc has been shown to be antagonistic in the rat (1–3) and in the chick (4). Molybdenum has been shown to exert an effect on copper metabolism of sheep (5) and of rats (2, 6–8) and iron has also been shown to be interrelated with copper metabolism (10–12).

The considerations which preceded the work reported herein were as follows. If the interrelationships are mediated at the gut level, it could be postulated that they are the result of interactions in or on the intestinal mucosa. Either alternative could indicate a common absorptive site for the Cu and the interrelated ion(s). The studies reported here were therefore undertaken in an attempt to determine the site or sites in the intestinal tract where Cu, Zn, Mo, and Fe, respectively, are most rapidly absorbed. Zinc and molybdate represent a cation and an anion, respectively, which interfere with copper metabolism (1–8), whereas, iron is an example of a cation that enhances the utilization of copper (10, 11).

EXPERIMENTAL

Rats of the Sprague-Dawley strain were used in these experiments. They were housed in stainless steel batteries with raised wire floors and were fed a stock chow diet.[2] This diet was found, by chemical analysis, to contain 7 to 10 ppm of copper, 45 ppm of zinc, 0.4 ppm of molybdenum, and 200 ppm of iron.

For individual experiments, the procedure was as follows. After an overnight fast, the rats were anesthetized with ether and the peritoneal cavity was opened by a 2.5- to 3.5-cm midline incision. The segments, with the exception of the stomach, were removed from the body cavity and ligated in 2 places. The second ligature was left loose, the needle was inserted through the ligature into the lumen of the gut, the ligature was tightened and the isotope used [3] was injected into the following segments: 1) stomach; 2) duodenum (about 0.5 to 7.5 cm distal to the pyloris); 3) mid-section (about 33 to 40 cm proximal to the ileocecal juncture); and 4) ileum (about 2 to 9 cm proximal to the ileocecal junction). The stomach was ligated at the pylorus but the esophagus was left open. After the injection had been made, the segment was returned to the

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[2] Big Red Dog Chow, Agway, Ithaca, New York. Trade names and company names are included for the benefit of the reader and do not infer any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.

[3] All isotopes were obtained from Union Carbide Nuclear Company, Oak Ridge, Tennessee.

body cavity and the incision was closed with sutures and stainless steel wound clips. After 2 hours for those animals that had received Mo^{99} and 3 hours for those animals that had received Cu^{64}, Zn^{65}, or Fe^{59}, the rats were anesthetized with ether, decapitated, and tissues were taken for direct gamma counting. Results are expressed as a percentage of the dose per tissue. Values for blood were arrived at by multiplying the counts per minute per gram of blood by 0.07 times the body weight of the animal, and values for kidneys were obtained by doubling the count obtained from one kidney. The data were subjected to an analysis of variance and means were compared using a multiple range test (13). Statements of significance are based on odds of at least 19 to 1 ($P \leqq 0.05$).

Details of dosage and other essential information will be given in conjunction with the results of the individual experiments to which they pertain.

RESULTS

Cu^{64} experiments. In these experiments, male rats weighing 250 to 400 g were used. The doses of copper ranged from 0.01 to 0.06 μmoles per rat because of differences in the specific activity of different batches of Cu^{64}. The isotope was received as $Cu(NO_3)_2$ in 1 N HNO_3. Upon receipt, it was diluted with physiological saline and the pH was adjusted to 5.0 to 6.0. Final dilution was to 10 ml, and each rat received 0.3 ml of the resulting solution. The results of these experiments are shown in table 1. The trend for the uptake of Cu^{64} from the various segments was as follows: stomach > duodenum > mid-section > ileum. This trend held regardless of which tissue was sampled and also held for the sums of the means of the sampled tissues. Statistical analyses indicated that all of the tissue uptakes were significantly higher ($P \leqq 0.05$) when the isotope was placed in the stomach than in the ileum. If the sums of the sampled tissues are used as an index of uptake, then absorption from the stomach is significantly higher than from the ileum at the 1% level of significance.

Zn^{65} experiments. In these experiments, male rats weighing from 200 to 350 g were used and these rats were allotted at random into replicates on the basis of body weight. The Zn^{65} was obtained as $ZnCl_2$ in 1 N HCl and had an initial specific activity of 7936 mc/g. Upon receipt, it was diluted with physiological saline and adjusted to pH 6.0. The final solution contained 0.3 μmoles of Zn/ml, and each rat received a dose of 0.15 ml of this solution. The results are presented in table 2. Analysis of the sums of the means for all the tissues reveals that Zn^{65} was taken up more quickly ($P \leqq 0.01$) from the duodenum than from any of the other segments. The tissue uptake from the ileum was higher ($P \leqq 0.05$) than from the stomach in all tissues except blood. Then, a rank of the means for total Zn^{65} uptake by the sampled tissues has the following order: duodenum > ileum > mid-section > stomach. Some difficulty was encountered in determining the exact dosages of Zn^{65}; hence the absolute values for tissue uptake are subject to this measurement error and are probably high. However, each rat received an identical dose; hence the relative rates of uptake from the different segments would be unaffected by errors in calculating the absolute dosage.

Mo^{99} experiments. Female rats with a weight range of 175 to 200 g were used in

TABLE 1

Uptake of Cu^{64} from various segments of the intestinal tract

	% of dose/tissue [1]				
Segment	Blood	Heart	Kidneys	Liver	Sum [2]
Stomach [3]	2.26^{a}	0.058^{a}	2.34^{a}	6.78^{a}	11.43^{a}
Duodenum	1.74^{ab}	0.042^{ab}	1.57^{ab}	4.52^{ab}	7.87^{ab}
Mid-section	1.37^{ab}	0.032^{ab}	1.18^{ab}	4.21^{ab}	6.80^{ab}
Ileum	0.53^{b}	0.014^{b}	0.31^{b}	1.45^{b}	2.33^{b}

[1] Each tissue value is the mean of 7 observations.
[2] Sum of the means for blood, heart, kidneys and liver.
[3] Values in any column not followed by the same letter are significantly different ($P \leqq 0.05$).

these studies. The Mo^{99} was obtained as NH_4MoO_4 and had an initial specific activity of 99 mc/g. Prior to use, it was diluted with physiological saline and the pH was adjusted to 7.0 with HCl. Each rat received 0.2 ml of the resulting solution which corresponds to 2.7 μmoles of Mo/rat. The results of these experiments are shown in table 3. The sum of the means shows that total uptake by these tissues from the stomach was lower ($P \leq 0.05$) than from any of the other segments. Uptake by the liver was greater ($P \leq 0.01$) from the duodenum than from the other segments, and uptake by the kidneys and the blood was higher ($P \leq 0.5$) from either the duodenum or the ileum than from the stomach. The means for uptake by the various tissues fall in the following order: duodenum > ileum > mid-section > stomach. An exception to this is blood where the mean for the ileum was slightly higher than that for the duodenum.

Fe^{59} experiments. Male rats weighing 250 to 400 g, and female rats weighing 175 to 250 g were used in these experiments. Rats were randomized into replications on the basis of sex and body weight. The Fe^{59} was received as $FeCl_3$ in 1 N HCl and had an initial specific activity of 25,454 mc/g. This original solution was diluted with physiological saline, and ascorbic acid was added to reduce the iron to the more soluble ferrous state. The final solution was adjusted to pH 6.0 with NaOH and contained 0.06 mmoles of ascorbic acid and 0.07 μmoles of iron/ml. Each rat received a dose of 0.15 ml. The results are presented in table 4. If the sums of the means of all the tissues are ranked,

TABLE 2

Uptake of Zn^{65} from various segments of the intestinal tract

	% of dose/tissue [1]				
Segment	Blood	Heart	Kidneys	Liver	Sum [2]
Stomach [3]	0.05^{b}	0.005^{c}	0.06^{c}	0.34^{c}	0.46^{c}
Duodenum [4]	5.41^{a}	0.525^{a}	7.78^{a}	38.09^{a}	51.80^{a}
Mid-section	0.66^{b}	0.063^{bc}	0.93^{bc}	5.61^{bc}	7.26^{bc}
Ileum	2.04^{b}	0.152^{b}	2.38^{b}	12.90^{b}	17.48^{b}

[1] Each value is the mean of 4 observations.
[2] Sum of the means for blood, heart, kidneys and liver.
[3] Values in any column not followed by the same letter are significantly different ($P \leq 0.05$).
[4] Only 3 observations in blood mean.

TABLE 3

Uptake of Mo^{99} from various segments of the intestinal tract

	% of dose/tissue [1]				
Segment	Blood	Heart	Kidneys	Liver	Sum [2]
Stomach [3]	1.99^{b}	0.046^{a}	0.44^{c}	1.12^{c}	3.60^{b}
Duodenum	4.91^{a}	0.397^{a}	1.34^{c}	2.82^{a}	9.46^{a}
Mid-section	3.99^{a}	0.098^{a}	0.81^{bc}	1.85^{b}	6.75^{a}
Ileum	5.17^{a}	0.115^{a}	0.98^{ab}	2.02^{b}	8.28^{a}

[1] Each value is the mean of 5 observations.
[2] Sum of the means for blood, heart, kidneys and liver.
[3] Values in any column not followed by the same letter are significantly different ($P \leq 0.05$).

TABLE 4

Uptake of Fe^{59} from various segments of the intestinal tract

	% of dose/tissue [1]					
Segment	Blood	Heart	Kidneys	Liver	Spleen	Sum [2]
Stomach [3]	0.52^{c}	0.007^{c}	0.34^{b}	0.17^{b}	0.02^{b}	1.06^{c}
Duodenum	6.34^{a}	0.064^{a}	1.15^{a}	1.35^{a}	0.16^{a}	9.06^{a}
Mid-section	2.28^{bc}	0.024^{bc}	1.00^{a}	0.58^{b}	0.13^{a}	4.01^{bc}
Ileum	3.11^{b}	0.044^{ab}	1.30^{a}	0.67^{b}	0.14^{a}	5.26^{b}

[1] Each value is the mean of 9 observations.
[2] Sum of the means for blood, heart, kidneys, liver and spleen.
[3] Values in any column not followed by the same letter are significantly different ($P \leq 0.05$).

they fall in the order: duodenum > ileum > mid-section > stomach. The total uptake by the sampled tissues was higher ($P \leqq 0.05$) from the duodenum than from any of the other segments and uptake from the ileum was higher than that from the stomach. The retention by individual tissues generally followed the same trend as the total uptake by all of the sampled tissues.

DISCUSSION

Copper was absorbed relatively quickly from the stomach with the absorption rate from the small intestine decreasing as the isotope was placed further away from the pylorus. The quantitative importance of the absorption of Cu^{64} from the stomach is difficult to evaluate, since it is not known how long a given portion of food remains in contact with the stomach under normal conditions. However, Cu^{++} was the only one of the cations tested which was absorbed from the stomach faster than from the intestinal segments. Sacks et al. (10) observed that when Cu was placed in an upper jejunal loop, serum copper values increased slightly but little or no difference was observed with distal or middle jejunal segments. Tompsett (14) postulated that Cu is absorbed from the upper part of the intestine where the contents are acid. However, to our knowledge the results reported herein are the first direct evidence for the absorption of Cu from the stomach.

For Zn, the duodenum had, by far, the highest rate of absorption, the ileum and the jejunem followed, in that order, and almost no Zn was absorbed from the stomach. Iron absorption followed a pattern qualitatively similar to that for Zn, namely, the duodenum and ileum were most active and little absorption occurred from the stomach. In an earlier study on the absorption of iron from various parts of the intestinal tract, Hahn et al. (17) noted that when radioiron was introduced into the terminal segment of a jejunal fistula, the amount absorbed was less than 50% of that retained when the dose was given in the stomach. This suggests that the greatest uptake occurs in the stomach and duodenum, but does not differentiate between them. Our results indicate that, of the two, the duodenum is the more important. With respect to Zn, there is little information on the major absorptive sites, but such information as is available indicates some similarity between the absorption of Zn and that of Fe. This agrees well with the results reported here.

Molybdenum was absorbed readily from both the stomach and the intestinal segments. The intestinal segments were more active in this respect than the stomach and little difference was observed between the various segments. Little information is available in the literature on the sites(s) of Mo absorption. However, the observation that all segments of the small intestine and, to a lesser extent, the stomach were functional with respect to absorption of the hexavalent Mo ion is consistent with reports of rapid absorption of the molybdate ion from the gut (15, 16).

Direct comparisons between the various isotopes are difficult to make because of widely different rates of absorption. This difficulty can be alleviated by comparing ratios rather than absolute values. Ratios were therefore calculated from the sums in tables 1 through 4. For each isotope, the ratio of uptake from a given segment of small intestine to uptake from the stomach was calculated and these are presented in table 5. This table serves to point up further the wide difference between the elements. The ratios range from less than one, in the case of Cu, to over one hundred for Zn. Zn^{65}, Mo^{99}, and Fe^{59} yielded qualitatively similar absorption patterns, namely, duodenum > ileum > mid-section > stomach; however, comparison of the ratios for these elements indicates that they are quantitatively different.

To recall briefly, the primary purpose of this work was to determine the part of the intestinal tract from which the selected isotopes were absorbed most rapidly. The

TABLE 5

Ratios of isotope uptake from the duodenum, the mid-section and the ileum to uptake from the stomach

Isotope	Ratio of isotope uptake		
	Duodenum-to-stomach	Mid-section-to-stomach	Ileum-to-stomach
Cu^{64}	0.7	0.6	0.2
Zn^{65}	112.6	15.8	38.0
Mo^{99}	2.6	1.9	2.3
Fe^{59}	8.5	3.8	5.0

results are relatively conclusive in this respect. It was also thought that these experiments might show whether the Zn-Cu, the Mo-Cu, or the Fe-Cu interrelations were mediated at the gut level. The results reported here do nothing to repudiate this idea, but a conclusive answer will require more direct experiments.

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Effect of High Phosphorus Intake on Calcium and Phosphorus Metabolism in Man [1]

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ABSTRACT The metabolic effects of phosphate added as glycerophosphate to the low and high calcium diet were studied in man under controlled dietary conditions. The increase in net absorption of phosphorus, upon addition of glycerophosphate, was greater during low calcium intake than during high calcium intake. Nevertheless, the improvement in phosphorus balance was similar during both intake levels of calcium since the urinary phosphorus excretion was higher during low calcium intake and the fecal phosphorus excretion was higher during high calcium intake. The high phosphorus intake led to a slight decrease in urinary calcium and to minor changes in fecal calcium excretion during both low and high calcium intake. The changes in calcium balance were only minor during the addition of phosphate to the low and high calcium diet, the calcium balances improving slightly during low calcium intake and remaining in the same range during high calcium intake. The simultaneous use of added amounts of calcium and of phosphate did not depress the intestinal absorption of either ion under the conditions of this study.

The calcium balance of patients with osteoporosis can be improved by the addition of calcium to the diet (1–5). However, this improvement is only slight (1–3), possibly due to the low absorption of calcium from the intestine in patients with osteoporosis (4, 5). Further improvement of the calcium balance by addition of phosphate to the high calcium intake has been suggested (6).

Several studies have been made on the effects of added phosphorus in children (7–9), and only few reports are available on adult man (10, 11). Two short-term studies on the effects of adding very large amounts of phosphate to a low calcium diet were carried out by Farquharson et al. (10); other studies have been made in 4 prisoners on the effect of different amounts of phosphate on calcium metabolism during the intake of intermediate amounts of calcium (11). In the present work, the interaction of calcium and phosphorus was investigated in man by studying the metabolic effects of increasing the calcium intake approximately tenfold while maintaining a constant phosphorus intake, and of increasing the phosphorus intake two- to threefold while maintaining a constant calcium intake.

EXPERIMENTAL

Seven patients were studied under controlled dietary conditions in our metabolic research ward. The age, sex and diagnoses of the patients and the type and duration of the studies are listed in table 1. The patients were ambulatory, in good nutritional state and had normal kidney function and normal gastrointestinal function. Patient 3 was asymptomatic except for slight back pain and was diagnosed as having osteoporosis; however, a bone marrow aspiration led to the diagnosis of multiple myeloma. The patients received a constant, analyzed low calcium-low phosphorus diet which contained from 1550 to 2330 kcal excepting patient 3 who consumed only 1200 kcal/day. The diet contained on the average 262 g carbohydrate, 60 g protein, 67 g fat, 202 mg calcium and 656 mg phosphorus per day. The composition of the diet is listed in table 2. The patients were maintained with the same constant diet in

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TABLE 1

List of patients studied

Patient	Age, sex	Diagnosis	Study	Study days
1	77, F	Osteoporosis	low calcium-low phosphorus	18
			low calcium-high phosphorus	18
			high calcium-low phosphorus	42
			high calcium-high phosphorus	20
2	70, F	Hypothyroidism	low calcium-low phosphorus	18
			low calcium-high phosphorus	18
			high calcium-low phosphorus	18
			high calcium-high phosphorus	20
3	70, F	Multiple myeloma	low calcium-low phosphorus	16
			low calcium-high phosphorus	20
4	41, M	Osteoarthritis	low calcium-low phosphorus	20
			low calcium-high phosphorus	24
			high calcium-low phosphorus	12
			high calcium-high phosphorus	30
5	39, M	Peripheral neuropathy [1]	low calcium-low phosphorus	36
			low calcium-high phosphorus	18
			high calcium-low phosphorus	18
			high calcium-high phosphorus	30
6	67, F	Osteoporosis	high calcium-low phosphorus	42
			high calcium-high phosphorus	30
7	43, M	Peripheral neuropathy [1]	high calcium-low phosphorus	42
			high calcium-high phosphorus	30

[1] Mild peripheral neuropathy secondary to previous chronic alcoholism.

TABLE 2

Composition of metabolic low calcium diet (1891 kcal)

	Carbohydrate	Protein	Fat
	g	g	g
Fruit juice, 200 ml	24		
Rice,[1] 20 g	16	} 15	
Bread, 100 g	52		
Spaghetti,[1] 20 g	15		
Jelly, 70 g	46		
Potatoes,[2] 100 g	19	} 5	
Canned vegetables, 200 g	18		
Sweetened canned fruit, 225 g	44		
Sugar, 28 g	28		
Meat (beef and turkey), 175 g		40	24
Butterfat, 50 g			41
Cream, 20 g			2
Total	262	60	67

[1] Weight of raw food. [2] Weight of cooked food.

the metabolic research ward from 3 to 12 months prior to the start of the study. Of the 7 patients, five were studied with a low calcium diet (patients 1–5), six with high calcium intake (patients 1,2,4–7) and four were studied during both low and high calcium intake (patients 1,2,4 and 5). In the high calcium study, the calcium intake was increased to an average of 1522 mg/day by adding calcium gluconate tablets to the low calcium diet, all other constituents of this diet remaining unchanged. During the high phosphorus studies, an average of 972 mg phosphorus as glycerophosphate was added to the low calcium control diet and an average of 893 mg phosphorus to the high calcium intake; this increased the phosphorus intake to an average of 1628 mg/day during the low calcium diet and to an average of 1549 mg/day during high calcium intake. The approximate Ca-to-P ratio of the low calcium-low phosphorus diet was 1:3, of the low calcium-high phosphorus diet 1:8, of the high calcium-low phosphorus diet 1:0.5 and of the high calcium-high phosphorus diet 1:1. The Ca-to-P ratio of 1:1 in the high calcium-high phosphorus diet was selected because these amounts of calcium and phosphorus are contained in the average American diet to which one quart of milk is added. To evaluate the

effects of phosphorus and of calcium alone, each level of these ions was tested separately, resulting in the other Ca-to-P ratios ranging from 1:0.5 to 1:8.

The body weight, the fluid intake and output, and the urinary excretion of calcium, phosphorus and creatinine were determined daily. Metabolic balances of calcium, phosphorus and nitrogen were determined for each patient before and during the addition of phosphorus to the low and high calcium diet. Aliquots of 6-day metabolic pools of urine and stool collections and representative aliquots of the diet were analyzed in each 6-day metabolic period.

The net absorption of phosphorus and calcium was calculated as follows:

$$\text{Net absorption } \% = \frac{\text{Intake} - \text{fecal excretion}}{\text{Intake}} \times 100.$$

Nitrogen was determined by a modification of the micro-Kjeldahl method described by Hawk et al. (12), phosphorus by the method of Fiske and SubbaRow (13) and calcium by a modification of the method of Shohl and Pedley (14). Stool calcium and phosphorus were determined on dry-ashed aliquots of 6-day metabolic pools.

RESULTS

Addition of phosphorus to the low calcium diet. Table 3 shows that the urinary calcium excretion of the 5 patients was low during the intake of the low calcium-low phosphorus diet. The calciuria ranged from 49 mg to 78 mg/day. The calcium balances of these patients were slightly negative in the control study and ranged from −35 mg to −170 mg/day. On addition of phosphorus to the low calcium diet, the urinary calcium excretion decreased in all patients except in patient 2. The changes in fecal calcium excretion were not consistent on addition of glycerophosphate to the low calcium diet. There was an increase in fecal calcium in patient 1, a slight decrease in patients 2, 3 and 5 and there was no change in patient 4. The calcium balance of patients 2, 3 and 5 improved slightly during phosphorus supplementation, the change in balance being 45 mg, 91 mg and 72 mg/day in the 3 patients, respectively, whereas the

TABLE 3

Mineral and protein balances of patients receiving added phosphorus (glycerophosphate) with low calcium intake

Patient	Study	No. days	Calcium				Phosphorus				Nitrogen balance
			Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance	
			mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
1	Control	18	140	49	175	− 84	519	345	210	− 36	+1010
	Glycerophosphate	18	146	34	199	− 87	1758	1336	325	+ 97	+1094
2	Control	18	186	53	168	− 35	668	328	261	+ 79	+2165
	Glycerophosphate	18	188	53	125	+ 10	1635	1094	232	+309	+2156
3	Control	16	140	64	191	−115	552	463	236	−147	− 962
	Glycerophosphate	20	145	36	133	− 24	1424	1192	297	− 65	+ 543
4	Control	20	272	78	308	−114	795	268	276	+251	+3078
	Glycerophosphate	24	259	65	304	−110	1674	762	463	+449	+2408
5	Control	36	271	57	384	−170	747	267	358	+122	+2202
	Glycerophosphate	18	258	38	318	− 98	1649	834	593	+222	+2295

calcium balances of patients 1 and 4 remained unchanged.

The urinary phosphorus excretion increased markedly in each patient during the addition of phosphate to the low calcium diet (table 3). The changes in fecal phosphorus excretion were less consistent: there was an increase in patients 1, 4 and 5, a slight increase in patient 3 and a very slight decrease in patient 2. The phosphorus balances of the 5 patients improved during the addition of glycerophosphate. The phosphorus balances became either more positive or less negative, the improvement in balance ranging from 82 to 230 mg/day.

Addition of phosphorus to the high calcium intake. Table 4 shows that the urinary calcium excretion ranged from 97 to 224 mg/day during the high calcium control study (low phosphorus intake). The calcium balances were positive during high calcium-low phosphorus intake in 5 patients, ranging from +105 mg to +476 mg/day, whereas the calcium balance was negative in one (patient 4). On addition of phosphate to the high calcium intake, the urinary calcium excretion decreased in the 6 patients, and the fecal calcium excretion was variable and increased in 2 patients (patients 2 and 7), remained unchanged in three (patients 4–6), and decreased in one (patient 1). The changes in the calcium balances were variable in the 6 patients during the addition of phosphorus to the high calcium intake. The calcium balance increased in patients 1 and 6, decreased in patients 2 and 7 and remained unchanged in patients 4 and 5, the average change in the calcium balance being not significant.

The addition of phosphate to the high calcium diet also resulted in a considerable increase in urinary phosphorus excretion (table 4). However, this increase was not as great as during the addition of phosphate to the low calcium intake. On the other hand, the stool phosphorus increased to a considerably greater extent during the addition of glycerophosphate to the high calcium intake than to the low calcium diet, except in patient 6. The phosphorus balance also improved in all patients in the high calcium-high phosphorus study except in patient 6 (table 4).

TABLE 4

Mineral and protein balances of patients receiving added phosphorus (glycerophosphate) with high calcium intake

Patient	Study	No. days	Calcium				Phosphorus				Nitrogen balance
			Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance	
			mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
1	Control	42	1483	97	1281	+105	519	266	225	+ 28	+ 652
	Glycerophosphate	20	1475	64	1193	+218	1419	591	628	+200	+1288
2	Control	18	1180	152	782	+246	678	244	296	+138	+2021
	Glycerophosphate	20	1181	123	962	+ 96	1383	568	498	+317	+2488
4	Control	12	1422	222	1380	−180	740	445	378	− 83	+ 404
	Glycerophosphate	30	1441	202	1416	−177	1717	704	864	+149	− 263
5	Control	18	1411	98	1122	+191	742	261	392	+ 89	+1804
	Glycerophosphate	30	1437	70	1127	+240	1695	635	771	+289	+2351
6	Control	42	1496	224	1152	+120	681	442	224	+ 15	+1077
	Glycerophosphate	30	1515	194	1118	+203	1422	1093	268	+ 61	+1455
7	Control	42	2111	127	1508	+476	748	239	262	+247	+2672
	Glycerophosphate	30	2080	86	1780	+214	1658	725	583	+350	+2150

Nitrogen balance was positive in all patients in the low and high calcium control studies except in patient 3 who received the lowest calorie intake. No definite trend in change of the nitrogen balances was noted on addition of phosphorus to either the low or high calcium intake (tables 3 and 4).

Table 5 summarizes the average changes in calcium and phosphorus balances during the addition of glycerophosphate to the low and high calcium diet. Prior to the addition of phosphorus to the low calcium diet, the average urinary phosphorus excretion was greater than the fecal phosphorus excretion, whereas with the addition of glycerophosphate, the urinary phosphorus excretion increased and was approximately 3 times as high as the fecal phosphorus. The addition of phosphate to the low calcium diet resulted only in a slight increase in fecal phosphorus. The average phosphorus balance of the 5 patients changed from +54 mg/day to +202 mg/day during the addition of glycerophosphate to the low calcium diet.

During high calcium intake the average urinary phosphorus excretion was about equal to the average fecal phosphorus, prior to the addition of phosphorus (table 5). With the addition of glycerophosphate, the urinary phosphorus increased by an average of 403 mg/day as compared with an increase of 710 mg/day during low calcium intake. In contrast, the fecal phosphorus increased to a greater extent on addition of phosphate to the high calcium intake than to the low calcium diet. This distribution in phosphorus excretion resulted only in a small change in the average urinary-to-stool phosphorus excretion ratio in the high calcium-high phosphorus study as compared with the high calcium-low phosphorus study. The average improvement of the phosphorus balances was similar, 148 mg and 156 mg/day, on addition of glycerophosphate to the low and high calcium diet, respectively.

The average changes in the calcium balances were minimal upon addition of glycerophosphate to the low and high calcium intake because of the minor changes in urinary and fecal calcium excretion during high phosphorus intake (table 5).

Changes in net absorption of phosphorus. Figure 1 shows the effect of glycerophosphate added to the low and high calcium intake on the net absorption of phosphorus. When glycerophosphate was added to the low calcium diet, the

TABLE 5

Averages of calcium and phosphorus balances and of net absorption during the addition of glycerophosphate

	Low calcium intake			High calcium intake		
	Without added phosphorus	With added phosphorus	Difference [1]	Without added phosphorus	With added phosphorus	Difference [1]
Calcium mg/day						
Intake	202	199	− 3	1517	1522	+ 5
Urinary	60	45	− 15	153	123	− 30
Fecal	245	216	− 29	1204	1266	+ 62
Balance	−104	− 62	+ 42	+160	+133	− 27
Net absorption, % [2]	− 25	− 7	18	21	17	− 4
Phosphorus mg/day						
Intake	656	1628	+972	685	1549	+864
Urinary	334	1044	+710	316	719	+403
Fecal	268	382	+114	296	602	+306
Balance	+ 54	+202	+148	+ 72	+228	+156
Net absorption, % [2]	59	77	18	57	62	5

[1] Difference between values obtained during the addition of phosphorus and during the low phosphorus control study.

[2] Net absorption calculated for each individual of the respective group which was then averaged. Negative net absorption indicates net loss.

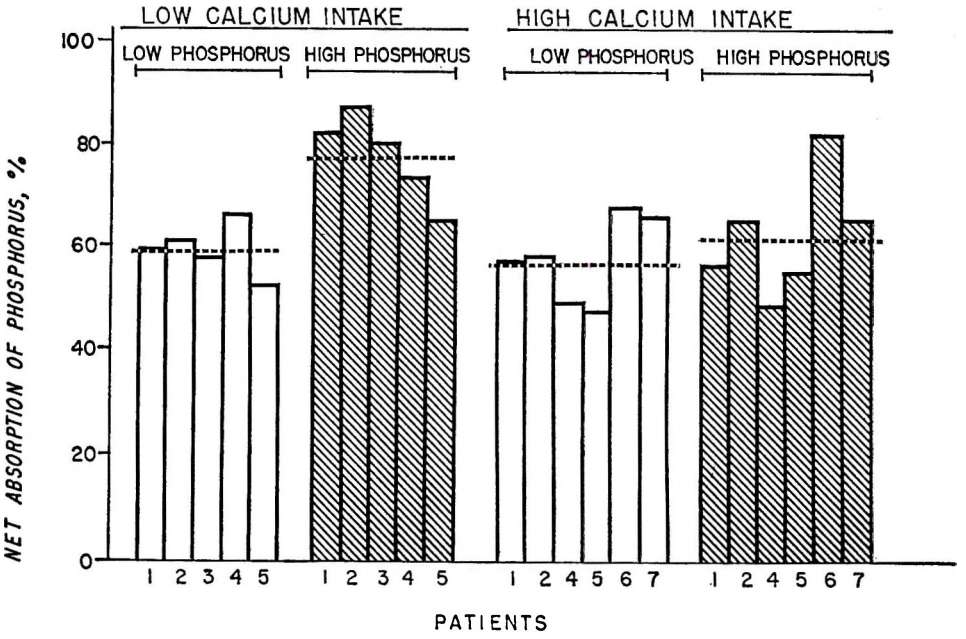

Fig. 1 Net absorption of phosphorus during low and high phosphorus intake. For calculation of net absorption see text (Experimental). Low calcium intake: Average calcium intake, 202 mg/day, average phosphorus intake, 656 mg/day. Average high phosphorus intake, 1628 mg/day. High calcium intake: Average calcium intake, 1517 mg/day, average phosphorus intake, 685 mg/day. Average high phosphorus intake, 1549 mg/day.

average net absorption of phosphorus, expressed as percentage of the phosphorus intake, increased significantly ($P < 0.01$), whereas this increase was not quite significant on addition of phosphate to the high calcium intake (P slightly > 0.1). Figure 1 also illustrates that the net absorption of phosphorus was similar with low and high calcium intake prior to the addition of glycerophosphate. The average changes in the net absorption of phosphorus during the intake of different levels of phosphorus and calcium are also listed in table 5.

DISCUSSION

Variable results have been reported on the effect of increasing the amounts of phosphate on calcium metabolism in experimental animals and man. Mellanby (15) observed that phosphates added to the diet in the form of phytates, decreased the absorption of calcium in vitamin D-deficient puppies. Bonting, as well as Nicolaysen, quoted by Malm (11), could not demonstrate changes in calcium absorption in rats, when different intake levels of phosphorus were used. In investigations in man, the fecal calcium was slightly increased in studies of college women when the phosphorus intake was increased from 800 mg to 1400 mg/day with a calcium intake of 1200 mg/day (16). Lutwak et al.[3,4] have recently reported the effect of different amounts of dietary phosphorus and calcium on the ratio of the retention of the 2 ions in children. These investigators performed balance studies on 2 groups of 10-year-old girls who received high levels of calcium and high levels of phosphorus in the diet, one group receiving 2.3 g calcium and 2.3 g phosphorus/day, the other group receiving 1.3 g calcium and 1.6 g phosphorus/day. Both the calcium and phosphorus balances were less positive with

[3] Lutwak, L. 1963 Interrelationships between calcium and phosphorus in the retention of mineral in man. Proceed. 6th International Congress on Nutrition, Edinburgh, Scotland, eds., C. F. Mills and R. Passmore. E. & S. Livingston Ltd., Edinburgh, P. 527.

[4] Lutwak, L., and G. D. Whedon 1963 Interrelationships between Ca and P balances in man. Clin. Res., *11*: 223 (abstract).

the intake of the higher amounts of calcium and of phosphorus than with the lower intake of both minerals. On the other hand, Widdowson et al. (9), in studies of breast-fed infants, reported that the addition of phosphorus increased the absorption of calcium and, at the same time, decreased the urinary calcium excretion resulting in improvement of the calcium balances. The phosphorus balances were increased to an even greater extent than the calcium balances, possibly reflecting the marked protein anabolism in infants.

The results obtained in the present study have shown that phosphorus is absorbed from the intestine to a much greater extent than calcium by adults during both low and high calcium intake.[5] The efficient absorption of phosphorus is indicated by the prompt and marked increase in urinary phosphorus excretion upon addition of phosphorus to the low calcium diet (tables 3 and 5). The high absorption of phosphorus could also be demonstrated with orally administered P^{32} in one of our patients who absorbed 95% of the dose of P^{32} during high calcium-low phosphorus intake.[6] The greater increase in urinary phosphorus excretion of 710 mg/day upon addition of phosphorus to the low calcium intake, as compared with the increase in urinary phosphorus excretion of 403 mg/day, upon addition of glycerophosphate to the high calcium intake, was due to the higher net absorption of the added phosphorus during low calcium intake than during high calcium intake (table 5). Despite the difference in net absorption of added phosphorus during the intake of different amounts of calcium (table 5), the improvement in the phosphorus balances of 148 mg and 156 mg/day, respectively, was similar during low and high calcium intake. These results can be explained by the higher urinary phosphorus excretion during low calcium intake and the higher fecal phosphorus excretion during high calcium intake (table 5).

The addition of phosphorus to the low and high calcium diet in the present study resulted in a slight decrease in urinary calcium excretion (tables 3–5), in agreement with results obtained by other investigators (7, 10, 11). Neither the slight increase in fecal phosphorus upon addition of glycerophosphate to the low calcium intake nor the more marked increase in fecal phosphorus during high calcium intake was accompanied by corresponding changes in fecal calcium excretion. The extent and type of formation of calcium salts in the intestine is not definitely known (17, 18), and calcium has been shown to be essential for the transport of phosphate across the intestinal wall.[7] In the present study, the calcium balances changed only slightly upon addition of relatively large amounts of phosphate to the low and high calcium diet as the changes in fecal and urinary calcium excretion were only minor. This lack of effect on calcium metabolism is similar to the results obtained in studies of added phosphate reported by Farquharson (10) and by Malm (11). Although the patients studied in the present investigation had different diagnoses (see table 1), the results obtained in this study are in good agreement with the data reported by Malm (11) in normal volunteers.

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Protein Metabolism in Livers of Chicks Fed Deficient-to-Excess Quantities of Protein and Lysine and Infected with Tuberculosis [1]

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ABSTRACT Protein metabolism was studied in individual livers of chicks conditioned from day of hatch with diets containing deficient-to-excess quantities of protein and L-lysine and infected with tuberculosis (*Mycobacterium avium*). Levels of total protein and the nucleic and free amino acids, used as parameters, were established and correlated with a TB involvement index based on morphological changes. Liver size increased with the intensity of the infection and with increasing dietary protein or lysine. This was attributed to an increase in protein anabolism. Cellular levels (in terms of DNA) of each of the 7 free amino acids observed decreased as liver size and the degree of infection increased, with lysine and arginine depressed to the greatest extent. The same effects were apparent when total quantities of free amino acids of the liver were calculated. Control or TB-infected chicks fed diets containing deficient or excess protein or lysine had generally lower cellular levels of free amino acids. In addition, the inoculated chicks conditioned with these same diets had a greater degree of tuberculous involvement compared with those fed diets with the normal amount of these nutrients.

Although our understanding of the relationship of diet to protein metabolism in a normal intact system has been steadily increasing, research on this phenomenon during infection is limited. Most work in this vital area has dealt with the effect of dietary changes on the course of infection and survival (1). Advances in the methodology of quantitating tissue amino acids provide a basis for more extensive studies. Since diet can influence the course of disease (1) then it is logical to assume that protein metabolism is implicated and that parameters selected specifically for estimating this process might reveal some of the interrelationships. A recent review of present concepts of mammalian protein metabolism (2) supports this assumption.

The effect of diet and Newcastle disease virus (NDV) on protein metabolism has been studied in susceptible chicks. An increase in nitrogen retention during the incubation period of the virus was found to be related to the effect of the NDV per se, and a decrease during the active involvement stage was attributed to the inanition caused by the disease (3). Later studies (4) showed that a depression in cellular levels of 7 free amino acids in the liver was related to the degree of NDV involvement, with lysine decreased to the greatest extent by the infection. A deficiency or excess of protein increased mortality and depleted amino acid pool levels in the liver of NDV-infected chicks to a greater extent than that observed in similarly infected birds fed normal amounts of protein (5).

Procedures worked out in the course of the foregoing trials have made it possible to extend observations to a bacterial model — *Mycobacterium avium*. Avian tuberculosis is characterized by a progressive loss of appetite and body weight, accompanied by an increase in liver and spleen size. The increase in size is associated with an increasing number of tubercles which tend to be limited to these 2 organs. Studies by Solotorovsky et al. (6) on chicks infected with *M. avium* have suggested that protein metabolism may be vitally affected. This was further indicated by the report of Wogan et al. (7) that the disease induced a serum hyperproteinemia with a decrease in albumin and increases in α_3-, β- and γ-globulin.

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Since the liver is one of the 2 principal sites of tuberculous involvement in the chick, the experiments reported here were undertaken to observe changes in liver protein metabolism in infected chicks conditioned from time of hatch with diets containing deficient-to-excessive quantities of protein and lysine. Levels of total protein, deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and 7 free amino acids were used as parameters of protein metabolism.

PROCEDURES AND RESULTS

All experiments used White Leghorn cockerel chicks of known breeding, housed in all-wire batteries in air conditioned rooms. Experimental diets and water were offered ad libitum from day one. At 10 days of age the chicks were divided into experimental groups on the basis of body weight and inoculated with *Mycobacterium avium*. Uninoculated birds (controls) were maintained for each trial.

In each experiment, growth and performance of the controls fed normal diets were compared with standard growth curves for similar experimental conditions to determine whether the chicks were attaining their genetic potential. Significant deviation ($< 5\%$) from the established standard was used to evaluate the possible presence of stress conditions that would warrant the discontinuance of the experiment. The studies reported herein met the required standards.

The infecting inocula, prepared from cultures maintained in the Rutgers Department of Bacteriology, were injected intracardially, 0.5 ml/bird. The chicks were weighed weekly and when the first statistically significant decrease in body weight occurred between treatment groups (usually 35 to 40 days post-inoculation) all birds were killed and the livers removed. The tissue sampling always took place between 9:00 and 11:00 AM (8). Liver sections of each bird were preserved in 10% formalin and Bouin's solution for histological study and the remainder of the liver was quick-frozen for subsequent biochemical analysis.

All determinations were made on individual livers. The DNA and RNA were determined by modifications of the Schmidt-Thannhauser method (9). Liver total protein was determined by the biuret technique, standardized by the Kjeldahl procedure, and the free amino acids were separated and quantitated by thin-layer chromatography (10).

Squibb (4) has pointed out the need for considering body size and degree of disease involvement between dietary treatment groups to avoid confounding interpretation of biochemical data. Therefore, the following formula was devised to serve as an estimation of the extent of the tuberculous process:

$$\text{TB index} = \frac{\text{Liver weight (g)} \times \text{no. tubercles}}{\text{Body weight (g)}}.$$

The number of tubercles was the average of those counted in 15 different low power ($100\times$) microscopic fields. Tubercles in this type of infection are essentially evenly distributed throughout the liver.

Data were analyzed statistically according to Snedecor (11). Procedures followed those applicable to factorial designs. Sums of squares were separated for individual degrees of freedom; tests of significance included least significant differences and F tests. To calculate total quantities of DNA, RNA, etc., the values in terms of milligrams per gram of liver were multiplied by liver weight. Cellular quantities were calculated from ratios to DNA, i.e., milligrams of RNA per milligrams of DNA.

Experiment 1. In this trial the effects of 4 strains of *M. avium* on liver protein, nucleic and free amino acids were studied and correlated with the TB index. All chicks were fed the same standard reference diet (12) throughout the trial. At 10 days of age they were assigned to 5 groups: group 1 served as controls, and groups 2 to 5 were inoculated with cultures of *M. avium*, designated RA, RB, KC and KT.

The TB index values (table 1) show that strain RA resulted in only a very slight infection. Strains KT and KC produced a moderate infection of about the same degree; the greatest involvement resulted from the RB strain. Liver weight and liver as the percentage of body weight increased significantly ($< 1\%$) with each significant increase in the TB involvement index.

The biochemical data are arranged (table 1) according to the degree of tuberculous involvement produced by the 4

TABLE 1

Relation of liver protein, nucleic and free amino acids to the degree of tuberculous involvement in chicks inoculated with 4 strains of Mycobacterium avium

Strain [1]	TB index	Liver wt	Liver	DNA	RNA	Protein	Lysine	Histidine	Arginine	Aspartic acid	Alanine	Valine	Leucines [2]
		g	*% body weight*	*mg/total liver*		*mg/total liver*		*mg/total liver*		*mg/total liver*		*mg/total liver*	
Controls	—	12.4	2.1	26.4	226	2924	16.5	8.3	14.0	18.7	18.6	9.5	15.2
Strain RA	0.03	15.3	2.6	27.4	269	3726	15.6	9.8	14.7	18.9	17.6	10.1	15.0
Strain KT	0.98	24.8	6.5	37.9	381	6151	5.4	7.5	6.4	22.1	9.5	6.1	7.0
Strain KC	1.16	22.4	5.3	37.6	350	5177	5.9	8.1	6.9	20.8	10.0	6.1	7.6
Strain RB	3.65	30.7	8.7	52.3	457	7240	3.0	6.7	4.8	20.7	9.8	5.8	4.6
Disease effect:	< 1%	< 1%	< 1%	< 1%	< 1%	< 1 %	< 1%	NS [3]	< 1%	NS	< 1%	< 1%	< 1%
				mg/g liver	*mg/mg DNA*		*mg/mg DNA*		*mg/mg DNA*	*mg/mg DNA*		*mg/mg DNA*	
Controls				2.15	8.8	113	0.64	0.33	0.55	0.72	0.71	0.37	0.59
Strain RA				1.81	10.0	138	0.58	0.36	0.56	0.70	0.67	0.38	0.56
Strain KT				1.53	10.3	166	0.21	0.24	0.23	0.64	0.30	0.19	0.24
Strain KC				1.69	9.6	142	0.18	0.22	0.21	0.57	0.28	0.16	0.21
Strain RB				1.75	8.7	137	0.06	0.13	0.09	0.38	0.19	0.11	0.09
Disease effect:				< 1%	NS	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%

[1] Arranged according to TB index values; 10 chicks/group.
[2] Leucine and isoleucine combined.
[3] NS indicates not significant.

strains; the difference between the cultures of strains KC and KT was not significant. Total quantities of DNA, RNA and protein increased linearly ($< 1\%$) with the TB index values. On the other hand, total quantities of lysine, arginine, alanine, valine and the leucines decreased linearly ($< 1\%$) as the severity of the infection increased. The slight reduction in histidine and increase in aspartic acid were not significant.

All 4 groups of infected chicks had significantly less ($< 1\%$) DNA per gram of tissue than controls, regardless of degree of infection. With the exception of the RNA of the RB group, the ratios of RNA and protein to DNA of all the infected birds were higher than those of the controls. There was a significant ($< 1\%$) linear decrease in the free amino acids/DNA as the TB index values increased with lysine, arginine, alanine and the leucines in terms of DNA decreased more than the others.

Experiment 2. This trial was designed to study the effect of deficient-to-excess quantities of dietary crude protein on liver protein metabolism. Isocaloric diets (5) containing 13.8, 21.3 and 41.3% crude protein were offered ad libitum. Two groups of chicks were used for each dietary level of protein: controls, and those inoculated with Rutgers KT strain of avian tuberculosis.

According to the TB index values the level of infection in this trial (table 2) averaged higher than in experiment 1. Compared with the chicks fed at a normal level of protein, there was a higher ($< 1\%$) degree of infection in those fed deficient and excess protein.

Liver weight increased linearly ($< 1\%$) with both increasing dietary protein and virulence of the infection. Liver as a percentage of body weight was greatest, irrespective of infection, in the groups fed a deficiency or an excess of protein.

Considering total quantities of each of the liver components determined, both diet and disease significantly increased ($< 1\%$) DNA, RNA and protein. With the exception of the RNA of the infected chicks fed excess protein, the increase was linear with the protein content of the diet. Diet significantly ($< 1\%$) affected all the free amino acids; lowest levels occurred in the 13.8% protein groups. Lysine and arginine were significantly ($< 1\%$) decreased by the infection, whereas aspartic acid, alanine and valine were significantly increased ($< 1\%$). There was no disease effect on total quantities of histidine and the leucines.

Statistical analysis of the data for diet effects showed that DNA per gram of liver was significantly lower ($< 5\%$) in the chicks fed the 13.8% protein diet. The RNA-to-DNA ratio decreased linearly ($< 5\%$) as the protein content of the diet increased. Diet effects on the free amino acids in terms of DNA were variable. Except for the arginine-to-DNA ratio, levels of the free amino acids were higher in the 13.8% protein group than in the 41.3% protein controls.

Tuberculosis significantly ($< 1\%$) depleted cellular levels of the amino acids except aspartic acid and alanine, with the extent of depletion greatest for lysine-to-DNA and arginine-to-DNA ratios.

Experiment 3. In this trial observations were made on the effect of 0.5 to 4 times the dietary requirement of L-lysine on liver protein metabolism of chicks infected with tuberculosis. The low lysine basal ration used has been described (13); this diet supplied all the known requirements of the chick except lysine which was calculated to be present at 0.5 of the requirement. Crystalline L-lysine [2] was then added to the basal diet to provide 0.5, 1, 2 and 4 times requirement. Two groups of chicks were maintained at each dietary level; controls and those inoculated with Rutgers KT strain of *M. avium*.

The level of tuberculous infection (table 3) resulting from the KT culture was considerably less in this trial than in experiment 2. Even so, the deficient (0.5) and excess ($4\times$) lysine groups had the highest TB index values. Lysine fed at 0.5 to 4 times dietary requirement had no effect on liver size of the control birds or on liver as a percentage of body weight. However, liver size of the infected birds increased linearly ($< 1\%$) with increasing lysine of the diet. Furthermore, liver as a percentage of body weight was greatest in the in-

[2] Supplied by Merck and Company, Rahway, New Jersey.

TABLE 2

Effect of dietary protein on liver protein, nucleic and free amino acids of chicks infected with Mycobacterium avium

Group [1]	Dietary protein	Treatment group	TB index	Liver wt	Liver	DNA	RNA	Protein	Lysine	Histidine	Arginine	Aspartic acid	Alanine	Valine	Leucines [2]
	%			g	% body weight	mg/total liver		mg/total liver		mg/total liver		mg/total liver		mg/total liver	
1	13.8	Control	—	15.3	4.1	24.0	200	2925	4.5	6.1	3.8	10.5	7.9	3.5	4.6
2	13.8	Infected	5.51	30.6	11.6	46.8	419	5899	1.9	3.2	1.5	17.5	10.6	4.0	3.3
3	21.3	Control	—	21.6	3.2	45.0	320	4512	12.2	10.0	7.8	18.4	9.6	6.0	8.2
4	21.3	Infected	3.58	51.5	10.1	99.1	732	11188	5.7	10.0	6.5	31.0	21.0	9.6	9.8
5	41.3	Control	—	30.1	4.1	63.1	410	6643	11.0	11.3	10.4	16.9	16.0	6.7	8.7
6	41.3	Infected	5.69	57.4	12.8	114.9	722	12522	4.5	11.7	5.4	26.8	22.9	10.3	7.8
Diet effect:			NS [3]	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%
Disease effect:			< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	NS	< 1%	< 1%	< 1%	< 1%	NS
						mg/g liver	mg/mg DNA	mg/mg DNA		mg/mg DNA		mg/mg DNA		mg/mg DNA	
1	13.8	Control				1.60	8.3	122	0.19	0.25	0.16	0.42	0.34	0.14	0.19
2	13.8	Infected				1.61	10.0	138	0.05	0.08	0.04	0.43	0.25	0.09	0.08
3	21.3	Control				2.08	7.7	105	0.28	0.23	0.19	0.44	0.23	0.14	0.20
4	21.3	Infected				1.96	8.0	122	0.06	0.10	0.07	0.33	0.22	0.10	0.11
5	41.3	Control				2.08	6.9	111	0.18	0.19	0.17	0.29	0.25	0.11	0.15
6	41.3	Infected				2.00	6.8	115	0.04	0.11	0.06	0.25	0.21	0.10	0.07
Diet effect:						< 5%	< 5%	NS	< 1%	NS	< 5%	< 5%	< 5%	NS	< 5%
Disease effect:						NS	NS	NS	< 1%	< 1%	< 1%	NS	NS	< 1%	< 1%

[1] Ten chicks/group.
[2] Leucine and isoleucine combined.
[3] NS indicates not significant.

TABLE 3

Effect of dietary lysine on liver protein, nucleic and free amino acids of chicks infected with Mycobacterium avium

Group [1]	Dietary lysine requirement	Treatment group	TB index	Liver wt	Liver	DNA	RNA	Protein	Lysine	Histidine	Arginine	Aspartic acid	Alanine	Valine	Leucines [2]
				g	*% body weight*	*mg/total liver*		*mg/total liver*		*mg/total liver*		*mg/total liver*		*mg/total liver*	
1	× 0.5	Control	—	12.1	2.6	24.4	166	2175	14.8	7.5	11.8	20.0	17.1	9.4	13.3
2	× 0.5	Infected	1.01	16.3	4.2	34.2	237	3387	10.2	6.0	9.0	19.0	17.8	9.0	12.1
3	× 1	Control	—	14.7	2.4	30.7	205	2975	18.9	10.2	15.2	33.3	18.6	14.9	19.9
4	× 1	Infected	0.48	17.2	3.2	33.1	237	3491	17.3	9.0	14.6	29.3	24.5	14.1	19.0
5	× 2	Control	—	14.3	2.4	27.7	206	2838	20.4	10.2	15.3	29.5	22.2	14.9	19.5
6	× 2	Infected	0.68	19.3	3.7	37.0	278	3640	15.7	8.5	13.2	27.1	21.3	12.9	17.1
7	× 4	Control	—	14.8	2.8	33.9	201	2764	18.8	8.9	12.3	23.1	21.0	11.3	15.8
8	× 4	Infected	1.32	21.6	4.4	42.1	311	4356	17.2	8.7	13.5	28.1	23.1	12.8	18.0
Diet effect:			< 1%	< 5%	< 5%	< 5%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 5%	< 1%	< 1%
Disease effect:			< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	NS [3]	< 5%	NS	NS	NS	NS
						mg/g liver	*mg/mg DNA*	*mg/mg DNA*		*mg/mg DNA*		*mg/mg DNA*		*mg/mg DNA*	
1	× 0.5	Control				2.02	7.1	95	0.64	0.33	0.51	0.88	0.73	0.42	0.57
2	× 0.5	Infected				2.02	7.8	111	0.35	0.21	0.31	0.64	0.57	0.30	0.41
3	× 1	Control				2.09	7.0	99	0.64	0.35	0.52	1.14	0.63	0.51	0.68
4	× 1	Infected				1.97	7.2	105	0.52	0.28	0.44	0.89	0.73	0.44	0.58
5	× 2	Control				1.94	7.8	106	0.79	0.40	0.59	1.17	0.89	0.58	0.76
6	× 2	Infected				1.94	7.7	102	0.44	0.23	0.36	0.75	0.60	0.36	0.48
7	× 4	Control				2.33	6.2	83	0.58	0.27	0.37	0.70	0.64	0.34	0.49
8	× 4	Infected				1.97	7.7	109	0.43	0.22	0.34	0.70	0.58	0.32	0.45
Diet effect:						NS	NS	NS	NS	NS	< 5%	< 5%	< 1%	< 1%	< 5%
Disease effect:						NS	NS	NS	< 1%	< 1%	< 1%	< 1%	NS	< 1%	< 1%

[1] Ten chicks/group.

[2] Leucine and isoleucine combined.

[3] NS indicates not significant.

fected groups fed 0.5 and 4 times dietary lysine requirement.

Statistical analysis for diet effects, irrespective of disease, showed: 1) total quantities of DNA ($< 5\%$), RNA and protein increased linearly ($< 1\%$) with the lysine content of the diet; 2) total quantities of all the free amino acids were lower ($< 1\%$) in the chicks fed 0.5 of the lysine requirement; and 3) except for lysine and alanine there was a tendency toward a linear reduction in total quantities of the other free amino acids as dietary lysine was increased from normal to 4 times requirement. Analysis for disease effects, irrespective of diet, showed that, compared with controls, total quantities of DNA, RNA and protein increased significantly ($< 1\%$) as a result of the infection. Of the free amino acids, the only significant changes were in total lysine and arginine which decreased ($< 1\%$, $< 5\%$).

The DNA per gram of liver was higher ($< 5\%$) in the control chicks fed excess lysine but there were no significant diet effects on the RNA-to-DNA or protein-to-DNA ratios. With the exception of alanine, all the free amino acids per DNA tended to be lower when the chicks were fed 4 times the requirement of dietary lysine. The tuberculous process had no effect on DNA per gram of liver, RNA-to-DNA or protein-to-DNA ratios, but cellular levels of the free amino acids were reduced in varying degrees, with the lysine-to-DNA ratio affected to the greatest extent.

DISCUSSION

This is one of a series of studies of protein metabolism during tuberculosis infection. The observations deal first with the liver since this vital organ is one of the principal sites of tuberculous involvement in the chick. Following present concepts of protein metabolism, levels of total protein, nucleic and 7 free amino acids (5 essential, 2 nonessential) were used as parameters which were correlated with dietary changes and the tuberculous process.

The results of these experiments confirm a working hypothesis that tuberculosis increases protein metabolism in infected livers and that the process is anabolic. Several data contributed to this confirmation. The experimental design called for killing control and infected birds at the time of a significant decrease in body weight between groups. The livers of the infected chicks were significantly larger than those of their controls, both in weight and in percentage of body weight. Although biochemical analyses confirmed an increase in tissue protein, other determinations made on representative samples showed control livers averaged 72% moisture and 4.0% fat, whereas infected livers averaged 78% moisture and 3.8% fat. Thus, neither fat nor moisture was responsible for the abnormal size.

The mechanism by which an infected liver starts to increase in size has not been defined. The abnormality no doubt is a reaction to the presence of the bacteria in the host. This is followed by degenerative changes of liver cord cells, an increase in leucocytes in the sinusoids, and an increasing prominence of the Kupffer cells. In the later stages the miliary tubercle forms, and an increase in the number and size of the hepatocytes follows.

The free amino acids, the *prima materia* for protein synthesis, are derived from the diet and labile reserves of carcass protein when the infection is slight. However, when the degree of tuberculous involvement is severe and inanition becomes acute, the demands of the enlarging liver come principally from body reserves. For example, unpublished data show significant depletion of the pectoral musculature. This apparent catabolism correlates with a serum hyperproteinemia that includes a decrease in albumin and increases in the globulin fractions (7).

In the trials reported here excessive quantities of lysine or protein also increased liver protein anabolism. Thus both diet and disease increased liver size, but there was a distinct difference in the mode of action. In the former there was no apparent inanition or muscle atrophy, whereas in the latter both of these conditions were present. The accelerated protein anabolism under the experimental conditions described herein strongly suggests involvement of the adrenal cortex. Unpublished data of these laboratories have shown that livers of cortisone-treated birds infected with NDV attain 5.7% of

body weight within a period of 3 days as compared with 2.8% for untreated controls. Corticosteroids are known to influence the body's protein anabolically or catabolically, depending on dosage. The action of the corticosteroids in increasing the catabolism of carcass protein (14) is followed by a transfer of amino acids to the liver and incorporation into protein (15, 16). This process is similar to that observed during the course of the tuberculous process.

The various parameters merit comment. As a group they follow present concepts and reflect the protein metabolism of a tissue in the normal state as well as the abnormal, which includes the biochemical contribution of the invading organism. Under disease stress conditions the changes in these parameters are of considerable magnitude (8).

The DNA as a point of reference provided the means for estimating cellular levels. This was important in these studies as total quantities of liver components did not indicate the complete biological picture due to the abnormal character of the tuberculous tissue. For example, total quantities of aspartic acid, alanine and valine were unaffected or increased by tuberculosis, whereas in terms of DNA, or at cellular levels, all the free amino acids were depressed. Increases in total quantities of DNA reflected changes in liver size as influenced by diet or disease. In these studies, DNA per gram of liver tissue was not significantly changed except when the diet contained excessive lysine or a deficiency of protein; the latter observation has been reported (5).

An increase in RNA synthesis generally resulted in an increase in total liver protein. Allison and co-workers (17) reported a fairly constant ratio between RNA and protein synthesis in the rat liver; they interpreted this to mean that there was a maximal amount of protein that could be formed and associated with each cell nucleus. We have noted this constancy in the livers of the noninfected controls fed balanced rations; the protein-to-RNA ratio averaged 14:1. However, with excessive dietary protein or a high TB index value the ratio increased significantly to 17:1. This increase in the protein-to-RNA ratio was attributed to a change in character of the liver tissue of which the inflammatory cells would be an example.

Quantitation of liver free amino acids served to provide an estimate of the availability of the *prima materia* of protein synthesis. Highest cellular levels were observed in those groups, infected or noninfected, that were conditioned from day of hatch with so-called balanced diets. Dietary deficiencies of protein or a single amino acid — lysine — resulted in cellular levels equal to or higher than those obtained from excessive protein or lysine diets. These results with imbalanced protein diets confirm a previous report for chicks (5). Recently Wannemacher and Allison (18) demonstrated that this phenomenon also occurs in the liver and muscle of rats fed varying levels of protein.

That 4 times the normal dietary requirement of lysine not only failed to increase cellular levels of this amino acid but tended to depress levels of the others, supports Christensen's statement (19) that "we should expect one group of amino acids to tend to be lost from the cells when a given amino acid is provided in sufficient excess. . ." Recently Jones (20) reported that excessive dietary lysine may induce an arginine deficiency in chick plasma and muscle. Calculation of each of the free amino acids presented here in terms of cellular (DNA) levels of lysine showed that not only arginine but all of the other amino acids were affected in the liver by excessive lysine.

With increasing tuberculous involvement the free amino acids of the liver decreased progressively, with the lowest cellular levels observed in the lysine and arginine components of the pool. The data do not indicate which occurred first, the depression of the amino acid pool or the reactive structural alterations produced by the infection. As with NDV (4, 5) the utilization was selective and of different magnitudes.

Although the 3 experiments were designed principally for within comparisons, other observations were made. As would be expected in an intact system, certain of the parameters showed both uniformity

and considerable variability between trials. This is reasonable since various sources and combinations of dietary proteins necessarily were incorporated in the test diets. Even though simple (exp. 3) to more complex (exps. 1 and 2) formulations were used, all were balanced, and produced growth rates that met genetic potential standards. With respect to the parameters, DNA per gram of tissue was similar for the noninfected control groups fed balanced rations. Departures from the control levels were interpreted to be indicative of changes in cell numbers per unit of tissue analyzed. For example, during NDV infection (4) DNA per gram of tissue increased apparently because the tissue became more compact due to inanition, thus increasing the number of cells per unit area. With tuberculosis infection, DNA was either less (exp. 1) as compared with that of controls, or remained the same (exps. 2 and 3). Differences in diet and tissue displacement, especially those related to quantity of inflammatory cells, would explain the variations between trials.

Hedgecock (21) reported that mice had greater resistance to tuberculosis when conditioned with diets containing 20% protein and that susceptibility increased when the diets were imbalanced by changing the protein content to 10 or 40%. The results reported here followed a similar pattern. As indicated by the TB index, greater involvement occurred in the groups fed deficient or excess quantities of protein or lysine.

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Influence of Dietary Lipids on the Fatty Acid Composition of Neutral Lipids and Phosphatides in Chick Liver and Bile [1]

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ABSTRACT Diets in which the fat content was derived from either cottonseed oil, linseed oil, rapeseed oil, a high linoleic acid margarine, butter, or a commercial stock diet were fed to one-week-old chicks for 6 weeks. An additional group of chicks was fed a fat-free diet. The influence of these diets on the fatty acyl content of hepatic and biliary phospholipids and non-phosphatide lipids was determined, and where possible, a direct comparison in the fatty acid content of a liver phosphatide was made with its biliary counterpart. The results demonstrated that phosphatidylethanolamine possessed distinctly different acyl groups in bile when compared with liver, and the differences were similar with all diets. Phosphatidylcholine in bile also had a different fatty acid content than the liver phosphatide but the differences were not as consistent as for phosphatidylethanolamine. Possible explanations for the alterations of the bile compounds are discussed. In general, the fatty acid content of the diet fed was reflected in the composition of liver and bile phospholipids although the patterns were, in some cases, complex to interpret.

Previous studies (1–3) in this laboratory on the relationship of diet to the composition of bile have led us to determine the influence of dietary lipids on the fatty acid patterns in the non-phosphatide [3] lipids and individual phosphatides of chick bile. In addition, the fatty acyl groups of individual phosphatides and non-phosphatide lipids from chick liver were analyzed and compared with the results obtained in the study of bile.

The role of phospholipids in facilitating the solubilization of cholesterol [4] has led investigators to identify the types of phosphatides in bile. Phillips (4) demonstrated the existence of lecithin, lysolecithin, cephalin and sphingomyelin in human bile, whereas Nakayama and Blomstrand (5) observed the same components in bile from rats and rabbits. Blomstrand (6) analyzed the fatty acids in the non-phosphatide lipid and total phospholipid fraction of human bile and observed a complex pattern of fatty acids in the lecithin fraction. A comparison between the fatty acids in human liver phospholipid and biliary phospholipid revealed sharp differences in fatty acyl groups (7).

METHODS

Experimental design. Five groups of one-week-old chicks were fed, for 6 weeks, a diet which consisted of the following: (in %) sucrose, 46.53; crude casein, 25; dried brewer's yeast, 10; fat, 10; salt mixture, 5.17; gelatin, 3; choline, 0.2; and a vitamin mixture, 0.1. The vitamin and salt mixture have been described in a previous communication (8). The salt mixture consisted of the following: secondary calcium phosphate, 2 H_2O, 2800 g; calcium carbonate, 875 g; dessicated magnesium sulfate, 404 g; potassium chloride, 460 g; sodium chloride, 500 g; ferric citrate (17.5% Fe), 100 g; manganese sulfate, 23 g; cupric sulfate, 5 H_2O, 2 g; zinc sulfate, 7 H_2O, 1 g; aluminum sulfate, 18 H_2O, 1 g; magnesium silicate, 1 g; di-

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[3] The term "non-phosphatide lipid" is used to denote those lipids removed from a silicic acid column by the use of chloroform. It is preferred to the term "neutral lipid" since this fraction does contain some free fatty acids.

[4] Isaksson, B. 1954 On the lipids and bile acids in normal and pathological bladder bile: A study of the main cholesterol dissolving components of human bile. Thesis, Lund, Sweden.

iodotyrosine, 1 g; cobalt carbonate, 0.05 g. The vitamin mixture was composed as follows: thiamine·HCl, 3 mg; riboflavin, 4 mg; nicotinic acid, 50 mg; Ca pantothenate, 12 mg; pyridoxine, 3.5 mg; biotin, 0.1 mg; folic acid, 2 mg; and sucrose, 925.4 mg. In addition to the above vitamin mixture, the diet also contained, for every 100 g, 1 mg of vitamin K substitute,[5] 10.0 mg of DL-α-tocopheryl acetate,[6] and 1.4 ppm of SeO_2. Vitamins A and D_3 were given as a solution, 0.1 ml twice a week per animal. This corresponded to an average of 250 IU of vitamin A and 20 IU of vitamin D_3/chick/day. The solution was made up from crystalline vitamin A acetate (1.0 g), crystalline vitamin D_3 (0.0058 g), 100 ml of ethyl alcohol, polyoxyethylene sorbitan monooleate[7] (64 g), and distilled water to make a total volume of 330 ml. The 10% fat in the diet was supplied to the 5 groups as: 1) cottonseed oil (CSO); 2) linseed oil (LSO); 3) rapeseed oil (RSO); 4) high linoleic acid margarine fat (MF); and 5) butter fat (BF). A sixth group (FF) was fed the fat-free basal diet, i.e., the above mentioned diet in which the 10% fat content was replaced by an equal amount of sucrose. A final group of chicks (CD) received a commercial diet which contained between 4 to 5% fat. The fatty acid compositions of the fats in the diets are shown in table 1. There were between 11 and 24 chicks in each of the 7 experimental groups.

At the end of the 6-week feeding periods, the chicks were decapitated and bladder bile was obtained from the individual groups by direct puncture of the gallbladder. By pooling samples, it was possible to obtain from 3 to 6 ml of bile in each group. The livers of the animals were also removed and combined in individual groups.

Extraction and purification of lipids. The livers from 10 animals in a group were cut into small cubes and mixed thoroughly in a beaker kept at 0°. From such a liver pulp, 7 g of tissue were taken and subjected to the extraction and purification procedures of Folch et al. (9). The total lipid fraction was then separated into a non-phosphatide lipid fraction and individual liver phosphatides by silicic acid column chromatography according to Glenn et al. (10). Monitoring of the silicic acid column was performed by the method of Bartlett (11).

Bile from the individual groups was treated in a similar manner except that the original extraction with chloroform: methanol (2:1 v/v) was lengthened to 30 minutes to insure a more complete extraction of lipid material.

[5] Synkavit, Hoffmann LaRoche and Company, Nutley, New Jersey.
[6] Ephynal Acetate, Hoffmann LaRoche and Company, Nutley, New Jersey.
[7] Tween 80, Atlas Powder Company, Wilmington, Delaware.

TABLE 1

The fatty acid composition of dietary fats

Fatty acid[1]	CD[2]	CSO	LSO	RSO	MF	BF
8:0	—	—	—	—	—	1.16[3]
10:0	—	—	—	—	tr	1.91
12:0	—	—	—	—	tr	2.65
14:0	tr	0.43	—	—	1.01	11.41
14:1	tr	—	—	—	—	2.03
16:0	15.74	19.86	5.80	4.10	25.73	33.60
16:1	1.47	—	—	—	—	3.82
18:0	2.25	2.21	4.70	1.50	4.83	11.90
18:1	23.95	18.43	19.51	15.30	27.84	27.58
18:2	52.79	57.94	15.17	21.95	40.60	2.86
18:3	3.80	1.13	54.00	14.21	tr	1.08
20:4	tr	—	—	3.48	—	—
22:1	—	—	0.82	42.90	—	—

[1] Indicates carbon length and unsaturation.
[2] CD indicates commercial stock diet; CSO, cottonseed oil; RSO, rapeseed oil; LSO, linseed oil; MF, margarine fat, and BF, butter fat.
[3] The amounts of fatty acids of 8:0 and lower are undoubtedly low because of high volatility of esters of these acids.

Analysis of fatty acids in lipid fractions. All analyses of fatty acid methyl esters were performed on a Podbielniak Chromacon Apparatus, using a diethylene glycol succinate phase (15.4% by weight, 80–100 mesh) coated on acid-washed Chromosorb W. Methylation of fatty acids was conducted by the method of Stoffel et al. (12), and identification of the fatty acid esters was by carbon number (13) and the use of standards obtained from the Hormel Institute, Austin, Minnesota. The fatty acid composition of each lipid fraction was determined by the triangulation method and the values reported are as area per cent. Linearity of detector response was checked periodically by analyses of standard mixtures of fatty acid methyl esters obtained from the Hormel Institute.

RESULTS

Separation of lipids in chick liver and bile.[8] The fractionation of phosphatides from liver and bile is shown in figure 1. Five phosphatides were identified in chick liver: cardiolipin, CL; phosphatidylethanolamine, PE; phosphatidylinositol, PI; phosphatidylserine, PS; and phosphatidylcholine, PC. A sixth phosphatide, sphingomyelin, could be eluted from the column with pure methanol but this component was not studied in this investigation. There were always appreciable amounts of PI and PC in PS, and since PS was not present in chick bile this phosphatide was not analyzed for its fatty acid content. The 2 major phosphatides in chick bile were PE and PC, although traces of CL, sphingomyelin and lysophosphatidylcholine could be detected by thin-layer chromatography. Because of the low concentration of these latter 3 phosphatides in bile, they were not studied in this investigation.

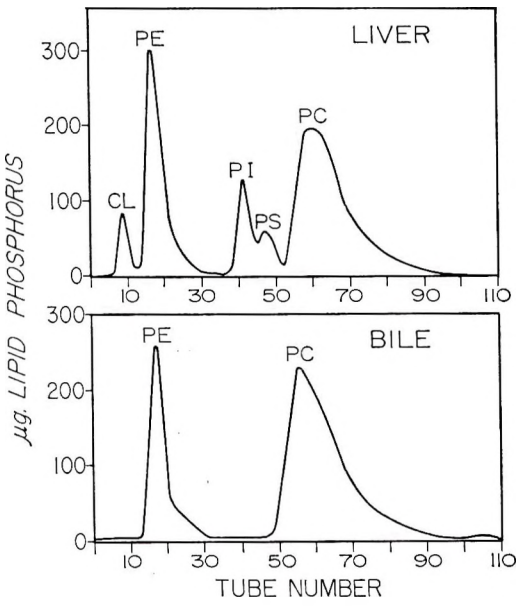

Fig. 1 Fractionation of liver and bile phosphatides by silicic acid column chromatography. Symbols: CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine and PC, phosphatidylcholine. CL and PE were eluted with chloroform:methanol (4:1, v/v), whereas PE, PS and PC were eluted with chloroform:methanol (3:2, v/v).

The composition of the phospholipid fractions from liver and bile, on the basis of the percentage of lipid phosphorus, is shown in table 2. The distribution of the individual phosphatides from livers of chicks fed the different diets exhibited some variation, but compared qualitatively with the composition of livers from other species although PE was somewhat higher and PC lower than in other animals. In bile, PC accounted for 76 to 86% of the lipid phosphorus, whereas PE content represented from 14 to 24%. The level of PE in chick bile was considerably higher than that reported in other species (14).

The non-phosphatide lipid fractions from liver and bile were not separated into their individual components, but the fatty acids in these fractions were methylated and analyzed by gas-liquid chromatography.

Fatty acids in chick liver cardiolipin. The fatty acid patterns of CL from livers of chicks fed the 7 experimental diets are listed in table 3. This phosphatide in chick liver, as in other species, was characterized by having high levels of linoleic acid. As a result of the commercial chick diet (CD), CL fatty acids contained 73% linoleic acid, and similar high levels of this acid were noted in CL from chicks fed the diets containing cottonseed oil (CSO),

[8] Abbreviations. The following abbreviations are used in this communication: CL, cardiolipin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; CD, commercial chick diet; CSO, cottonseed oil diet; LSO, linseed oil diet; RSO, rapeseed oil diet; MF, high linoleic acid margarine fat diet; BF, butter fat diet and FF, fat-free diet.

TABLE 2

Percentage composition [1] of phospholipids in liver and bile from chicks fed different diets

Diet [2]	Liver					Bile	
	CL [3]	PE	PI	PS	PC	PE	PC
CD	3.4	29.9	8.5	5.2	52.8	24.1	75.9
CSO	6.1	37.7	10.8	5.0	40.4	14.4	85.6
RSO	7.3	34.7	8.5	2.5	46.8	14.6	85.4
LSO	3.2	35.3	11.7	4.2	45.6	20.1	79.9
FF	2.6	37.1	13.4	3.0	43.9	19.1	80.9
MF	2.7	33.4	12.1	3.5	48.3	23.6	76.4
BF	4.6	34.4	9.8	2.9	48.3	20.0	80.0

[1] Values represent percentage of total lipid phosphorus in phospholipid fraction from liver or bile, with the exception of sphingomyelin which was not isolated.
[2] CD indicates commercial stock diet; CSO, diet contained 10% cottonseed oil; RSO, diet contained 10% rapeseed oil; LSO, diet contained 10% linseed oil; FF, diet contained no fat; MF, diet contained 10% margarine fat; and BF, diet contained 10% butter fat.
[3] CL indicates cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; and PC, phosphatidylcholine.

TABLE 3

Fatty acid composition of chick liver cardiolipin

Diet	CD [1]	CSO	LSO	RSO	MF	BF	FF
Fatty acid							
14:0	—	tr	tr	tr	tr	0.50	tr
14:1	tr [2]	tr	tr	tr	tr	tr	tr
16:0	5.85	8.76	4.23	2.03	3.07	1.87	4.00
16:1	3.80	1.65	1.70	1.24	1.02	18.16	31.72
17:0	tr	—	—	—	tr	0.29	—
18:0	4.24	5.71	2.08	1.22	2.72	0.35	1.86
18:1	9.94	9.98	8.33	8.35	7.88	25.93	48.37
18:2	72.95	63.58	68.56	76.62	80.30	47.72	11.72
18:3	tr	1.02	9.60	3.54	0.71	1.23	1.12
20:2	—	4.61	1.01	3.47	3.04	0.58	tr
20:3	1.54	1.44	1.07	1.40	—	2.84	1.21
20:4	1.68	3.26	3.41	2.11	1.26	0.51	tr

[1] CD indicates commercial chick diet; CSO, diet contained 10% cottonseed oil; LSO, diet contained 10% linseed oil; RSO, diet contained 10% rapeseed oil; MF, diet contained 10% margarine fat; BF, diet contained 10% butter fat; and FF, diet was fat-free.
[2] Fatty acid content too low to be measured.

TABLE 4

Fatty acid composition of chick liver phosphatidylinositol

Diet	CD [1]	CSO	LSO	RSO	MF	BF	FF
Fatty acid							
14:0	—	tr	tr	tr	—	0.44	tr
14:1	—	tr	tr	tr	—	—	tr
16:0	10.41	6.68	2.48	6.48	4.59	7.07	7.44
16:1	2.36	0.43	0.37	tr	0.47	4.03	12.40
18:0	37.73	49.07	42.86	46.57	33.08	42.65	34.68
18:1	8.14	4.29	4.09	3.71	5.54	14.58	25.68
18:2	16.63	18.74	22.84	10.98	31.87	12.62	3.64
18:3	tr	tr	2.76	0.80	—	—	0.34
20:2	1.40	0.62	tr	—	1.49	—	1.20
20:3	6.07	1.29	3.41	4.82	2.30	10.23	12.98
20:4	15.32	18.89	8.18	21.45	20.66	8.38	1.63
20:5	—	tr	3.90	1.43	—	—	—
22:4	1.93	tr	tr	—	—	—	—
22:5	—	tr	6.57	1.24	—	—	—
22:6	—	tr	2.54	2.60	—	—	—

[1] Abbreviations described in table 3.

TABLE 5

Fatty acid composition of phosphatidylethanolamine from chick liver and chick bile

Diet	CD [1]		CSO		LSO		RSO		MF		BF		FF	
	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile
Fatty acid														
14:0	tr	tr	0.3	tr	tr	—	tr	—	tr	0.2	tr	tr	tr	tr
14:1	tr	tr	1.1	tr	0.9	tr	tr	tr	0.9	0.3	tr	0.5	1.0	tr
15:0	—	—	—	—	—	—	1.0	—	0.9	—	1.0	—	—	
16:0	17.0	22.6	11.2	18.8	9.6	16.3	13.9	21.9	12.5	19.3	13.0	22.8	10.9	14.4
16:1	2.6	2.4	tr	1.6	tr	1.3	1.0	1.1	0.4	0.8	0.7	1.9	3.3	10.6
17:0	tr	tr	1.3	—	1.2	—	1.1	—	1.0	0.2	0.7	tr	0.6	—
18:0	32.6	23.1	38.8	32.3	42.3	34.6	32.5	28.1	34.5	30.1	33.7	24.3	32.6	15.8
18:1	8.0	9.1	2.3	5.1	4.5	6.1	4.3	6.9	3.8	6.8	8.4	13.6	24.3	45.7
18:2	12.4	16.5	11.2	21.8	7.7	9.8	7.8	13.5	12.4	19.3	4.6	8.2	2.2	3.7
18:3	tr	1.2	—	—	2.5	5.2	1.5	2.1	tr	tr	0.4	1.0	0.6	—
20:3	2.0	0.6	—	—	tr	—	—	—	tr	0.6	3.9	3.5	17.6	9.9
20:4	18.8	11.7	23.6	12.3	5.2	2.1	18.8	12.7	25.4	13.9	15.1	7.6	5.8	tr
20:5	0.9	—	2.1	—	12.4	10.4	2.8	tr	—	—	3.7	3.1	1.1	—
22:3	—	1.2	—	—	—	—	—	—	1.4	1.2	1.5	—	—	—
22:4	tr	1.1	4.5	1.3	—	—	—	—	4.2	5.2	1.2	—	—	—
22:5	tr	1.0	tr	3.5	2.5	2.7	2.0	tr	tr	—	1.6	1.9	—	—
22:6	5.7	9.5	3.5	3.3	11.2	11.6	13.2	13.7	2.5	2.2	10.6	11.6	tr	—

[1] Abbreviations described in table 3

TABLE 6

Fatty acid composition of phosphatidylcholine from chick liver and chick bile

Diet	CD [1]		CSO		LSO		RSO		MF		BF		FF	
	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile
Fatty acid														
14:0	tr	tr	0.2	—	tr	—	tr	—	tr	tr	0.2	0.7	tr	tr
14:1	tr	tr	0.2	tr	tr	tr	tr	tr	—	tr	tr	0.1	tr	tr
16:0	28.4	27.8	25.5	28.7	20.3	20.9	28.6	30.2	28.3	33.6	28.1	24.3	26.6	23.6
16:1	2.7	4.9	1.2	3.2	tr	3.2	1.8	3.2	1.8	2.4	3.8	5.9	6.5	10.9
18:0	23.6	17.4	25.8	20.7	34.8	28.6	24.2	16.5	20.3	17.8	23.0	18.8	18.1	10.9
18:1	16.2	17.0	6.7	7.6	7.7	10.7	9.4	15.4	8.9	8.9	25.1	29.0	42.4	46.7
18:2	19.0	19.3	21.2	28.0	17.5	19.9	17.7	19.8	22.3	23.7	8.3	9.6	1.0	3.5
18:3	tr	0.9	tr	—	2.1	4.2	1.5	1.7	0.6	tr	—	0.5	—	—
20:3	1.4	1.4	0.3	—	—	—	—	0.7	—	1.3	3.9	3.0	5.4	4.3
20:4	5.5	5.7	15.0	11.8	3.3	1.7	9.8	6.9	15.8	10.1	4.3	2.7	tr	tr
20:5	tr	0.7	—	—	6.7	7.3	—	1.2	—	—	1.5	2.2	—	—
22:3	tr	0.4	—	—	—	—	—	—	—	—	—	—	—	—
22:4	1.1	0.7	3.0	tr	—	—	—	—	2.0	1.4	—	—	—	—
22:5	tr	1.2	—	—	2.3	tr	—	—	—	—	—	tr	—	—
22:6	2.1	2.9	—	—	5.3	3.4	7.1	4.3	—	0.7	1.8	3.3	—	tr

[1] Abbreviations described in table 3.

linseed oil (LSO), rapeseed oil (RSO) and margarine fat (MF). The CL from the animals fed the fat-free diet contained only 12% linoleic acid, whereas the chicks receiving butter fat had 48% of this acid. In both of these groups, the decrease [9] in linoleic acid was accompanied by increased levels of oleic acid and palmitoleic acid, with the greatest increases occurring in animals fed the fat-free diet. Although the livers from animals receiving the FF or BF diet had high levels of eicosatrienoic acid there was little incorporation of this acid into CL.

When the chicks were fed the LSO diet which contained high amounts (54%) of linolenic acid, there was a large increase of this acid in liver CL, but none of the longer chain polyunsaturated fatty acids to which linolenic acid can give rise were incorporated into the molecule.

Fatty acids in chick liver phosphatidylinositol. The fatty acid compositions of this component in the livers of chicks receiving the different diets are shown in table 4. As in other species (15) PI was characterized by a high amount of stearic acid and a tendency to accumulate appreciable levels of linoleic acid and arachidonic acid. With the FF diet, the amounts of linoleic and arachidonic acids were low and the content of oleic acid, palmitoleic acid and eicosatrienoic acid high. Similar changes, but of a lesser magnitude, were observed in PI from chicks receiving the BF diet. In the animals receiving the LSO diet, the arachidonic acid content of PI decreased even though the linoleic acid content of the molecule was increased. The LSO diet did not lead to appreciable incorporation of linolenic acid but did lead to the presence of eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid in PI. These 3 longer chain polyunsaturated acids were also present in PI when the chicks received the RSO diet which contained approximately 14% linolenic acid.

Fatty acids in chick liver and bile phosphatidylethanolamine. The effects of the 7 diets on the fatty acyl composition of PE in both liver and in bile are presented

[9] In the ensuing presentation of Results, the words "increased" or "decreased" are used to compare fatty acid levels with those values obtained with the CD diet, unless otherwise stated.

TABLE 7

Fatty acid composition of non-phosphatide lipid from chick liver and chick bile

Diet	CD [1]		CSO		LSO		RSO		MF		BF		FF	
	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile
Fatty acid														
14:0	tr	tr	0.5	—	tr	—	tr	—	tr	tr	2.0	—	tr	—
14:1	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	—
16:0	37.8	30.0	28.9	32.8	15.1	21.9	26.1	25.7	29.1	24.1	34.7	28.5	24.5	27.6
16:1	6.1	7.0	2.7	10.1	2.9	5.5	3.7	6.9	2.2	6.1	5.9	7.1	8.7	17.1
17:0	tr	1.2	—	—	—	—	—	—	tr	0.9	—	0.4	—	—
18:0	10.9	12.6	12.6	13.1	17.7	13.2	10.8	12.6	12.1	13.8	10.3	12.6	9.0	4.0
18:1	30.6	34.1	19.2	19.4	26.5	25.0	24.6	26.0	23.5	22.7	41.1	41.8	52.5	45.4
18:2	8.9	15.0	29.6	20.6	12.7	16.1	17.3	18.5	27.3	21.7	4.0	4.9	1.9	5.4
18:3	2.1	tr	0.5	—	19.4	9.1	5.7	4.7	tr	2.6	0.7	2.8	—	—
20:2	—	—	—	—	—	—	—	—	—	—	—	—	1.1	—
20:3	tr	—	tr	—	0.5	—	tr	—	—	—	—	0.8	2.2	tr
20:4	3.6	—	6.1	4.0	1.9	4.7	11.8	5.7	5.9	7.3	1.4	1.2	—	—
20:5	—	—	—	—	3.7	—	—	—	—	—	—	—	—	—
22:3	—	—	—	—	1.1	—	—	—	—	—	—	—	—	—
22:4	—	—	—	—	—	—	—	—	tr	—	—	—	—	—
22:5	—	—	—	—	1.6	—	—	—	—	—	—	—	—	—
22:6	—	—	—	—	1.5	—	—	—	—	—	—	—	—	—

[1] Abbreviations described in table 3.

in table 5. Since PE was present in bile in a high concentration, it was possible to compare the fatty acids in this phosphatide with the PE obtained from liver. The fatty acid composition of bile PE was different from liver PE and certain interesting relationships were demonstrated. First, with all diets the arachidonic acid content of bile PE was considerably lower than in liver PE and this was always accompanied by an increase in linoleic acid and oleic acid in bile PE when compared with liver PE. Whereas this inverse relationship existed in the unsaturated acid content of PE, a similar relationship existed for the major saturated acids present in this molecule. The stearic acid level in bile PE was consistently lower than in liver PE, whereas the reverse was true for palmitic acid, i.e., palmitic acid of bile PE was consistently higher than it was in liver PE.

The specific effects of the diets on the acyl content of this phosphatide were as follows. The FF diet produced the expected decrease in linoleic acid, arachidonic acid and docosahexaenoic acid in both liver and bile, and these decreases were accompanied by large increases in oleic acid and eicosatrienoic acid. The LSO diet produced a marked decrease in arachidonic acid and led to increases in eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid. Both the CSO and MF diets, which contained high amounts of linoleic acid, resulted in increased amounts of arachidonic acid in PE but did not affect the level of linoleic acid in the molecule. The RSO diet produced an increase in docosahexaenoic acid content of both liver and bile PE.

Fatty acids in chick liver and bile phosphatidylcholine. A comparison in the fatty acyl content of hepatic and biliary PC from chicks fed all the experimental diets is presented in table 6. When the chicks were fed the commercial stock diet, there was little difference between the fatty acids in PC from liver and bile. With all of the other diets, the relationship between linoleic acid and arachidonic acid content in liver and bile PC was similar to that observed in the case of PE, but it was not as pronounced. Namely, the linoleic acid content of PC in bile increased, whereas the level of arachidonic acid decreased when compared with liver PC. The stearic acid content of bile PC was consistently lower than in liver PC, but there was no consistent relationship between palmitic acid in liver and bile PC.

The fat-free diet resulted in sharp decreases in the content of linoleic acid and arachidonic acid in PC, with an accompanying large elevation in oleic acid. As with PE, the feeding of the LSO diet led to decreased levels of linoleic acid and arachidonic acid in PC, whereas linolenic acid and products of linolenic acid metabolism, particularly eicosapentaenoic acid, were incorporated into the molecule. Note that the feeding of the CSO and MF diets, which were rich in linoleic acid, resulted in a greater relative increase of arachidonic acid into PC than of linoleic acid itself.

Fatty acids in chick liver and bile nonphosphatide lipids. The variations in the fatty acyl patterns of these lipids in liver and bile from chicks fed the experimental diets are shown in table 7. Except for the observation that palmitoleic acid was consistently higher in bile than in liver nonphosphatide lipids, there was no other consistent difference between the fatty acid composition in liver and bile. To the contrary, the fatty acids noted in both liver and bile were rather closely related and reflected the diet which was fed. For example, large increases were observed in linoleic acid content in chicks fed the CSO and MF diets. When the LSO diet was fed, high amounts of linolenic acid and products of linolenic acid were observed in the non-phosphatide lipids of chick liver but none of the latter were observed in chick bile. The FF diet caused a sharp reduction in linoleic acid content, and arachidonic acid was no longer present in this lipid fraction. With this diet, oleic acid content of the non-phosphatide lipids increased to very high levels in both liver and bile. The BF diet showed similar, but not as marked changes compared with the chicks receiving the FF diet.

DISCUSSION

Numerous studies have been concerned with the role of dietary lipids on the fatty acid composition of adipose tissue, hepatic tissue and plasma, but little is known as

to the effects of ingested lipids on the fatty acyl pattern in bile lipids (6). The present study was designed to determine what effects 7 different diets would have on the lipid composition of chick bile. Since it is believed that bile lipids are derived from the liver, the fatty acid composition of individual liver phosphatides and the non-phosphatide lipid fraction of liver were analyzed and, where possible, compared with their counterparts in bile.

It was possible to compare hepatic and bilary phosphatidylethanolamines, phosphatidylcholines, and non-phosphatide lipids since all of these compounds were present in sufficient concentration in bile, but the low level of other biliary phosphatides prevented a direct comparison with their hepatic counterparts. An analysis of the fatty acid content of biliary PE demonstrated clearly that it was different from that of liver PE. Furthermore, definite relationships between certain fatty acids in these molecules could be ascertained. First, the arachidonic acid content of bile PE was always considerably lower than that of hepatic PE, whereas the linoleic acid and oleic levels in bile PE were always higher than in liver PE. This inverse relationship of unsaturated acids was observed regardless of the diet fed to the chicks. A similar reproducible inverse relationship existed between stearic acid and palmitic acid in PE of liver and bile, namely, the stearic acid content of bile PE was always lower than liver PE, whereas the inverse was true in the case of palmitic acid. There are at least 2 ways to interpret these observations. First, they could mean that only a discrete portion of liver PE was secreted into the bile. The fraction in the bile would thus represent the PE from liver which had relatively higher amounts of linoleic acid, oleic acid and palmitic acid. If this were the case, then an analysis of plasma PE might show the opposite of that observed in bile, namely, plasma PE should have higher arachidonic acid and stearic acid but lower linoleic acid, oleic acid and palmitic acid than in liver PE. Unfortunately the fatty acid composition of plasma PE was not analyzed in this study. A second explanation to account for the different acyl patterns in liver and bile PE could be that a sufficient number of liver PE molecules are modified in some way, possibly by transesterification mechanisms during the secretory process, to yield the acyl pattern observed in bile. It is possible that a certain acyl pattern of bile PE is required to perform its function which may be concerned with the solubilization of cholesterol in bile. An analysis of the fatty acids in liver and biliary PC generally supported the results for PE, but the differences were not as consistent.

The results obtained in the analysis of bile and liver phosphatides support the generalization that the fatty acyl patterns of these compounds are dependent upon the type of fat in the diet, although the effects produced, are in some cases complex to interpret. For example, the feeding of the LSO diet, with its high linolenic acid content, resulted in a greater incorporation of this acid in liver and bile phosphatides and non-phosphatide lipids. There were, however, large increases in the products of linolenic acid metabolism (eicosapentaenoic, docosapentaenoic and docosahexaenoic acid) in the lipids. Although these increases occurred, there were, with the minor exception of CL, sharp decreases in the levels of arachidonic acid in the phosphatides and non-phosphatide lipids, even though in some cases the levels of linoleic acid were increased. The cause of this is not known but the results could support the hypothesis of Rahm and Holman (16) that high levels of linolenic acid suppress arachidonic acid formation by competing with linoleic acid for the enzyme site which initiates the conversion of linoleic acid to arachidonic acid. However, it is possible that one of the products of linolenic acid metabolism, such as eicosapentaenoic acid, is responsible for the decreased formation of arachidonic acid.

The feeding of the FF diet consistently led to sharp decreases in the linoleic acid, arachidonic acid and docosahexaenoic acid levels of the phosphatides. Under such conditions, the chicks attempted to maintain unsaturation in these compounds by incorporating greater amounts of oleic acid, palmitoleic acid and 5,8,11-eicosatrienoic acid. At the present time, it can not be ascertained whether oleic acid is converted to 5,8,11-eicosatrienoic acid by the same enzyme system which converts

TABLE 8

Percentage of unsaturated fatty acids in liver and bile PE [1] and PC

Diet	CD [1]		CSO		LSO		RSO		MF		BF		FF	
	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver	Bile	Liver
Phosphatide [2]														
PE	54.2	50.4	51.9	48.3	49.2	46.9	50.0	51.4	50.3	51.0	53.5	51.7	69.9	55.9
PC	53.9	47.0	50.6	47.5	50.4	45.2	53.3	47.2	48.5	51.4	56.2	48.7	65.4	55.3

[1] Abbreviations described in table 3.
[2] PE, phosphatidylethanolamine; PC, phosphatidylcholine.

linoleic acid to arachidonic acid. It appears that in vitro experiments with the isolated enzymes are needed to prove or disprove this point. The BF diet generally produced similar results as the FF diet but of a lower magnitude. This probably reflects the low content of linoleic acid in butter fat.

Although the type of dietary lipid played an important role in determining which fatty acids were present in liver and bile lipids, the degree of unsaturation in the individual phosphatides was fairly consistent. For example, in table 8, the percentages of total unsaturated fatty acids in liver and bile PE and PC, with the exception of bile from animals fed the fat-free diet, were close to 50%. The observation that approximately one-half of these 2 phosphatides contained unsaturated acids may be due to the fact that the β-position of certain phosphatides is occupied primarily by unsaturated fatty acids.

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Study of Purified Diets for Growth and Reproduction of the Ruminant [1,2]

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ABSTRACT Two purified diets were compared for growth of lambs. Diet 25p contained primarily casein as the source of nitrogen and diet 27v contained primarily urea. Although the average gain and feed efficiency were higher for diet 25p, differences were not statistically significant. Diet 25p was also shown to sustain normal reproduction of ewes. From results of the present report and previous studies, the thesis is developed that 5% alfalfa in the casein-containing purified diets we have developed furnishes a factor(s) required for reproduction of ewes but not for growth of lambs.

Previous investigations (1) showed that the growth of lambs fed a purified diet containing 20% casein (diet 17) was not improved by the addition of 5% alfalfa. In contrast, when urea was substituted for the source of nitrogen, the addition of 5% alfalfa improved the growth response of lambs markedly. The growth response of lambs fed the urea-alfalfa-containing diet, however, was not equal to that of the lambs fed the preformed protein-containing diet number 17. Since B-vitamins had been added to diet 17 and omitted from the urea-alfalfa diet, the possibility that differences obtained between these 2 diets were due to the presence or absence of B-vitamins could not be ruled out.

Concurrently with the growth experiments, the adequacy of diet 17 for reproduction of ewes was also being tested. The results of the reproduction study carried out over a period of 2 years indicated that diet 17 did not sustain normal reproduction.[3] Vigorous rams from the University flock showed little interest in servicing the ewes fed the purified diet. Several ewes that became pregnant carried their fetus almost to term before the fetus died in utero. Attempts to settle ewes by the artificial insemination technique [4] also were unsuccessful.

With these results in mind, the present study was undertaken with 2 primary objectives. The first was concerned with whether or not the purified diet containing urea and alfalfa could be made equal to the preformed protein diet for growth of lambs by the addition of B-vitamins and methionine. The second objective was to determine if the addition of alfalfa to the preformed protein diet would sustain normal reproduction of ewes.

EXPERIMENTAL

The details of the biological procedures used in the growth trial were similar to those described previously (1). Twelve lambs approximately 2.5 to 3 months of age were divided into 6 pairs based on weight, and then each pair was assigned at random to the 2 experimental diets (table 1).[5] The experimental period was 33 weeks.

In the interest of equalizing the experimental diets as nearly as possible the casein content of diet 17 (1) was reduced from 20 to 12% (diet 25p. table 1) to match the nitrogen content of the urea in

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[3] Unpublished data, G. Matrone, 1962.

[4] Dickey, J. F. 1961 The use of progesterone and pregnant mare serum (PMS) to synchronize estrus prior to artificial insemination in sheep. Master's thesis, Department of Animal Science, N. C. State of the University of North Carolina at Raleigh, Raleigh, North Carolina.

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TABLE 1

Composition of experimental diets

	Diet 25p	Diet 27v
	%	%
Casein	12.0	0.0
Urea	0.0	4.6
Glucose [1]	22.1	29.5
Starch	30.6	30.6
Hydrogenated vegetable fat [2]	4.0	4.0
Cellulose	3.0	3.0
Alfalfa leaf meal [3]	5.0	5.0
Vitamin mix [4]	5.0	5.0
$KHCO_3$	4.0	4.0
$NaHCO_3$	6.0	6.0
$CaCO_3$	1.0	1.0
$CaHPO_4$	1.8	1.8
Mineral mix [5]	3.0	3.0
Methionine mix [6]	2.5	2.5

[1] Crystalline glucose. Corn Products Sales Company, Norfolk, Virginia.

[2] Primex B and C (pure vegetable shortening), Procter and Gamble Company, Cincinnati.

[3] Alfalfa leaf meal (20% protein). National Alfalfa Dehydrating and Milling Company, Kansas City, Missouri.

[4] Vitamin mixture/45.36 kg diet: thiamine·HCl, 400 mg; riboflavin, 850 mg; nicotinic acid, 1.13 g; Ca pantothenate, 1.42 g; pyridoxine·HCl, 570 mg; folic acid, 57 mg; p-aminobenzoic acid, 1.13 g; inositol, 11.35 g; biotin, 11.4 mg; choline chloride, 113.45 g; menadione (2 methyl-naphthoquinone), 115 mg; 0.1% vitamin B_{12} (with mannitol), 4.66 g; α-tocopheryl acetate, 570 mg; glucose, 2132 g. In addition, 4000 IU of vitamin A and 400 IU of vitamin D/day/45.36 kg body weight were administered by capsule.

[5] Mineral mixture/45.36 kg diet: KCl, 273 g; NaCl, 239 g; $MgSO_4$, 204 g; $CuSO_4 \cdot 5H_2O$, 893 mg; $FeSO_4 \cdot 2H_2O$, 7648 mg; $MnSO_4 \cdot H_2O$, 1399 mg; ZnO, 2263 mg; $CoCO_3$, 9 mg; KI, 6 mg; Na_2MoO_4, 9.6 mg; glucose, 633 g.

[6] Methionine mix contained 4 parts glucose/1 part methionine and furnished 0.5% methionine to total diet.

the contrasting diet (diet 27v, table 1). In addition 5% alfalfa and 0.5% DL-methionine were added to each diet. Diet 25p was also fed to the ewes in the reproduction study.

The sampling procedure and analysis of blood during the growth experiment were as described previously (1). At the end of the growth trial, 2 separate runs for rumen fluid determinations were made. In the first run samples were taken by stomach tube from 6 animals of 3 replications of the growth trial and for the second run, samples were taken via rumen fistula from 4 of these animals. The animals were force-fed 227 g of diet in one liter of water. In each run a rumen sample was taken before feeding and subsequent samples were taken at 0.75, 1.5, 3 and 6 hours after feeding. Volatile fatty acid analysis of the rumen samples by gas chromatography and pH determinations were made as described previously (1). The procedures used for the nitrogen balance study with 6 of the experimental animals at the end of the growth trial have been described elsewhere (2).

The 10 ewes in the reproduction study were animals previously used in growth studies with purified diets. Thus all ewes in the experiment had been raised on a purified regimen from the time they were 2.5 to 3 months old. They had been removed from the growth trials at one year of age; during the second year (1961–1962) they were fed diet 17 in the reproduction study mentioned earlier. At the beginning of this reproduction study (1962–1963), the ewes were approximately 2 years of age.

The ewes were housed in pens in groups of two or three. Each day the ewes were turned out into a cindered exercise lot for 3 or 4 hours. The cinders were residue of bituminous coal used in the heating plant of the University. A ram obtained from the University flock and fed a normal hay-grain diet was turned out with the ewes in the exercise lot.

RESULTS AND DISCUSSION

The average gains and feed intakes of the lambs in the growth experiment are shown in table 2. Although, on the average, the animals fed the casein-containing diet, 25p, gained more and the kilograms of feed per kilogram gain was lower than for those lambs fed diet 27v, the differences were not statistically significant. The average daily gains (table 2) obtained in this study are comparable to those obtained with diet 17 in the previous

TABLE 2

Comparison of mean gains and feed intakes of lambs fed purified diets containing primarily casein or urea as the main source of nitrogen

Criteria [1]	Diets	
	25p (casein)	27v (urea)
Initial wt, kg	15.9	15.5
Gains, kg [2]	35.0	31.6
Avg daily gain, kg	0.160	0.145
Kg feed/kg gain	7.82	8.47

[1] Each value is an average of 6 animals.

[2] Standard deviation of a mean is ± 1.77 kg. Experimental period of 33 weeks for 5 pairs of lambs and 22 weeks for one pair.

study (1). The average daily gain obtained with diet 27v, the alfalfa-urea-containing diet, was superior to that obtained in the previous study (1) with similar urea-containing diets, one, diet 26, in which alfalfa was omitted, and another, diet 27, in which only the B-vitamins were omitted. Presumably, both vitamins and alfalfa supplements are required. It appears, however, that by proper supplementation, the purified diet 27v, containing primarily urea nitrogen, is comparable for growth of lambs to one (diet 25p) containing primarily preformed protein nitrogen.

The data obtained from the nitrogen balance study are shown in table 3. Analysis of the diets showed that actual nitrogen content of diet 25p was less, 1.88%, than that of diet 27v, 2.27%. Thus, since feed intake during the nitrogen balance study was equalized, the lambs fed the urea-containing diet, 27v, took in more nitrogen. The average nitrogen retention, however, was quite similar. More nitrogen was absorbed by the animals fed the urea-containing diet, but these animals also showed a higher excretion of nitrogen in the urine. Since the nitrogen intakes were different, assessment of the urinary nitrogen excretion cannot be made. The dry-matter digestibilities of the 2 diets were high and comparable to each other, i.e., 86.5% for diet 25p and 87.1% for diet 27v.

TABLE 3

Data from nitrogen balance study

Criteria [1]	Diets	
	25p (casein)	27v (urea)
Nitrogen in diet, %	1.88	2.27
Fecal nitrogen, g	24.17	21.82
Urinary nitrogen, g	38.53	56.58
Nitrogen balance, g	+22.56	+24.62

[1] Each value is a mean of 3 animals for a collection period of 5 days.

The volatile fatty acid data expressed as percentage of total acid are shown in figure 1. Marked differences in the volatile fatty acid patterns between diets from sampling period to sampling period were not obtained. In general the ratio of ruminal acetic to propionic acid tended to be higher for the lambs fed diet 27v than for those fed diet 25p. Isovaleric acid, how-

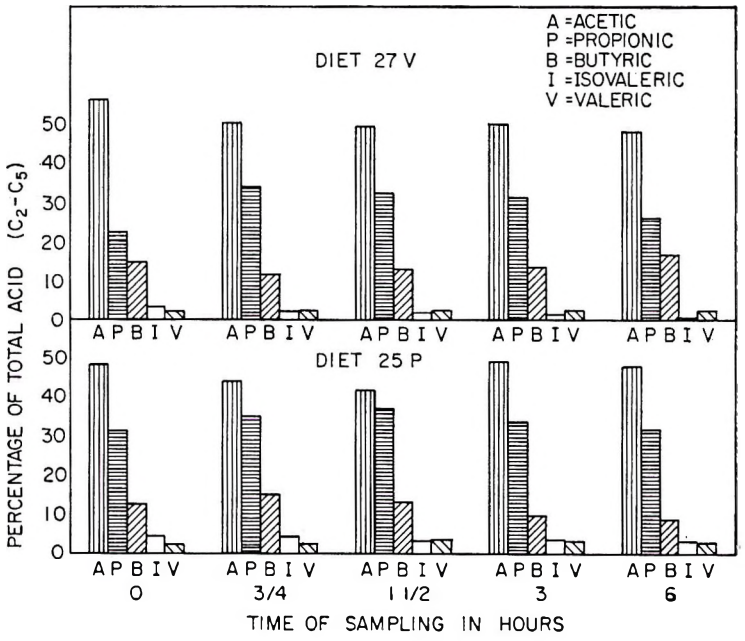

Fig. 1 Volatile fatty acids in samples of rumen fluid of lambs fed the experimental diets 25p (casein) and 27v (urea). Each bar graph is a mean of 5 animals.

ever, was significantly lower ($P \leqq 0.05$) for diet 27v than for diet 25p when all sampling periods are considered. This trend is consistent with that obtained in a previous study (1) in which isovaleric acid was found to be lower for lambs fed purified diets containing primarily urea nitrogen in contrast to those containing protein nitrogen. The results are in harmony with the concept that the branched-chain acids arise chiefly as end products of protein degradation (3).

The mean pH values of rumen samples plotted against time of sampling are shown in figure 2. The pH of the rumen samples taken at 0.75 hours and 1.5 hours were significantly lower ($P \leqq 0.05$) for the animals fed diet 25p than for those fed diet 27v. Presumably, ammonia released from the urea in diet 27v increased the buffering capacity of the rumen.

Data on serum calcium, phosphorus, magnesium, sodium and potassium are not presented since no marked differences between diets were obtained and because the values were similar to those reported previously (1).

The results from 9 of the 10 ewes fed diet 25p in the reproduction study are shown in table 4. The tenth ewe suffered a broken leg before reaching term. A Caesarean operation revealed 2 dead fetuses. The 9 ewes gave birth to 15 lambs. Two were born dead and two died several hours after birth. Thus the lambing percentage fell well within the normal range. The lambs were left with their dams for approximately 6 weeks. During this period the lambs also had access to the diet of their dams. Since the gains of the lambs were comparable to normally raised lambs, presumably the lactation levels of the dams were adequate during the 6-week period.

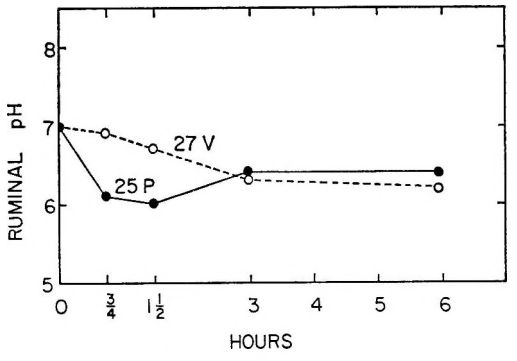

Fig. 2 The pH of samples of rumen fluid of lambs fed experimental diets 25p (casein) and 27v (urea). Each bar graph is a mean of 5 animals.

TABLE 4

Reproduction study — lambs born, weight at birth and sex

Ewe no.[1]	Ewe wt [2]	No. lambs born	Wt of lambs	
			Born alive	Born dead
	kg		*kg*	*kg*
317	65.8	1	♀ 5.5	
293	60.3	2	♂ 4.3 [3]	
			♂ 3.8	
319	60.8	1	♀ 4.4	
315	59.4	2	♂ 4.6	♂ 2.5
356	62.1	2	♂ 4.5	
			♀ 3.6	
304	68.5	1	♀ 4.9	
291	59.0	2	♂ 2.9 [4]	♂ 2.5
311	61.2	2	♂ 4.3	
			♂ 3.8	
320	61.7	2	♂ 4.5	
			♀ 3.2	

[1] Ten ewes, 2 years of age, received diet 25p in the reproduction study. One female ewe broke its leg; Caesarean operation revealed 2 dead fetuses almost to term.
[2] Weight before pregnancy.
[3] Breech birth, died several hours after birth.
[4] Died several hours after birth.

It seems unlikely that the positive results obtained in this reproduction study in contrast to the negative results obtained previously were due to an inorganic element picked up in the cindered exercise lot, first, because of the extensive number of mineral elements supplied by the diet and second, because of the results from the subsequent (1963–1964) reproduction study. In the 1963–1964 study alfalfa was deleted from diet 25p, and again no live lambs were obtained from 8 ewes even though they had access to the cindered exercise lot. The pretreatment of these ewes and breeding management were similar to those of the previous experiment.

Five per cent alfalfa is the basic difference between diet 25p, which sustained reproduction, and diet 17 (1), which has been found to be inadequate for reproduction. It seems reasonable to conclude, therefore, that the alfalfa meal contains a factor(s) for reproduction. Presumably, the alfalfa supplied a factor(s) which

affected the ewes directly or through the microorganisms in the rumen.

From the data presented in this report and those reported previously (1), it is concluded that diet 25p is adequate for both growth and reproduction, whereas diet 17 (1), which is similar except for the omission of alfalfa, has been shown to be adequate and equal only for growth of lambs. Whether or not diet 27v, which contains primarily urea nitrogen, can sustain reproduction is now under consideration.

ACKNOWLEDGMENT

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Iron, Copper, and Manganese in Germfree and Conventional Rats [1,2]

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ABSTRACT The effects of the microflora on the metabolism of iron, copper, and manganese were studied using germfree and conventional rats of both sexes. Hemoglobin and hematocrit values were similar in both groups, whereas plasma iron and copper levels were slightly but significantly lower in germfree than in conventional rats. The liver of the germfree rat stored more iron than that of the conventional rat, but the spleen, kidneys, and the total body of the germfree rat contained less iron than those of the conventional animal. As expected, the iron levels in females were higher than in males in both experimental groups. The relative proportions of ferritin and hemosiderin iron in the various organs of germfree and conventional rats were comparable. The levels of copper in these organs and in the total body followed the same pattern as the iron levels in germfree and in conventional rats. In contrast with the observations on iron and copper, the concentrations of manganese in the above mentioned organs and in the total body were similar in germfree and conventional rats. The results suggest a lower rate of iron and copper metabolism and a similar rate of manganese metabolism in germfree rats as compared with conventional rats.

Germfree rabbits in our laboratory demonstrated bone fragility, hind leg paralysis, irregular pregnancies, and low hemoglobin and plasma iron levels, whereas none of these symptoms occurred in their conventional counterparts maintained with the same sterilized diet containing recommended levels of nutrients. Similar symptoms have been linked to a dietary deficiency or impaired absorption of some trace minerals such as iron, copper, manganese and zinc (1). Observations of this nature indicated the possibility of an effect of the microbial status on the metabolism of these minerals. No reports about the trace mineral metabolism in germfree animals were available.

Since these symptoms are associated mainly with one or more of the above trace minerals (1), the present study was undertaken as an initial step to determine the effects of the microflora on the iron, copper, and manganese status of rats as determined by tissue and total body analysis. Furthermore, the relative proportions of ferritin iron and hemosiderin iron in the tissues of germfree and conventional rats were investigated, as it has been shown that a number of physiological and pathological factors affect the relative proportions of iron stored as ferritin and hemosiderin (2). Rats were used in this investigation because of the unavailability of an adequate number of germfree rabbits at the time of the study.

MATERIAL AND METHODS

Germfree and conventional albino rats of Lobund stock (Wistar origin) were used in this investigation. The germfree and conventional animals were genetically closely related. The germfree rats were housed in the Reyniers germfree system as described previously (3), and the conventional rats in the open colony room. Following weaning, all rats were maintained with steam sterilized, fortified practical-type diet L-462, which contained 800 ppm of iron, 100 ppm of copper and 200 ppm of manganese (4). Food and water were supplied ad libitum. Twenty rats (10 males and 10 females) of each category (germfree and conventional) were used for tissue analysis. Six female rats of each category were used for the fractionation of tissue iron.

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The animals were killed at 14 weeks of age. To avoid diurnal variation effects, all animals were killed at the same time of day throughout the experimental period. Rats intended for tissue analysis were anesthetized with ether. Blood was collected from the heart into a heparinized syringe and the residual blood removed as much as possible by bleeding freely from the carotid arteries. Liver, spleen and kidneys were removed, blotted to remove traces of blood, and weighed. Animals intended for total body analysis were killed by anesthetizing with ether. The gastrointestinal tract was removed, freed of its contents and washed in deionized water. The entire carcass and the washed gastrointestinal tract were used for analysis. Care was taken to minimize the loss of blood.

Hemoglobin was determined as oxyhemoglobin (5) using 0.02 ml of heparinized blood and 5 ml of 0.007 N NH_4OH and measuring the absorbency of the resulting solution at 550 mμ in a Coleman spectrophotometer. The hematocrit was measured by a macromethod after centrifuging the heparinized blood at $1200 \times g$ for one hour (6).

Iron, copper, and manganese content was determined after wet-ashing of the samples. For carcass analysis, the entire animal with the washed gastrointestinal tract was cut into pieces in an Erlenmeyer flask containing 60 ml of concentrated HNO_3 and 10 ml of 60% $HClO_4$. After the carcass had completely disintegrated overnight, the flask was heated on a sand bath. Portions of concentrated HNO_3 were added periodically until the solution was clear. Heating was continued until white fumes of $HClO_4$ were given off. A similar procedure was adopted for the wet-ashing of spleen, liver, and kidney samples, using smaller quantities of acids. The clear digest was diluted and measured aliquots were used for analysis.

Plasma iron was measured by the macromethod of Ramsay (7) as modified by Scarlata and Moore (8). The same method was used for the determination of total iron in the aliquots of digest of total carcass, liver, spleen, and kidney. For the 6 germfree and 6 conventional females used for the fractionation of liver, spleen and kidney iron, the method described by Kaldor (2) was adopted to separate total tissue iron into heme iron and storage (non-heme) iron. The heme iron was measured by the method of Kaldor (2). The storage (non-heme) iron was measured as described by Kaldor (9) with the following modifications. Iron was extracted from homogenized tissue with 4% HCl for 30 minutes at 95°. The supernatant solution was deproteinized with 40% trichloroacetic acid and the iron content determined spectrophotometrically with alpha, alpha-dipyridyl reagent (8) instead of ortho-phenanthroline reagent. Storage iron was further separated into water-soluble ferritin iron and water-insoluble hemosiderin iron. The iron content of the water-soluble and water-insoluble portions of homogenized tissue centrifuged at $2000 \times g$, representing ferritin and hemosiderin iron respectively, was measured by the method of Kaldor (2) again using alpha, alpha-dipyridyl reagent instead of ortho-phenanthroline.

The copper content of plasma and of tissue digest was measured by the method of Rice (10) using oxalyldihydrazide in HCl and a saturated solution of concentrated NH_4OH with oxalyldihydrazide, respectively. The manganese content of tissue digest was measured by the new benzidine procedure of Sastry et al. (11). To remove HNO_3 and $HClO_4$, which otherwise would interfere with the assay of manganese, a measured amount of tissue digest was heated at 100° to dryness and dissolved with deionized water.

The data were analyzed by analysis of variance techniques as outlined by Steel and Torrie (12).

RESULTS

Total body, liver, spleen and kidney weights of germfree and conventional rats at 14 weeks of age are summarized in table 1. Because the germfree animals accumulated large amounts of material in the cecum, the body weights were corrected for cecal contents in all groups. The liver, spleen, kidney and corrected body weights of conventional rats were higher than those of germfree rats. When these organ weights were calculated per 100 g corrected body weight, there was no difference in spleen and kidney weights

TABLE 1

Body and organ weights of germfree and conventional rats at 14 weeks of age

	Germfree		Conventional		Significance (*P* values)	
	Male	Female	Male	Female	Status [1]	Sex [2]
Body wt,[3] g	263 ± 5 [4] (20) [5]	167 ± 2 (20)	289 ± 4 (20)	196 ± 3 (20)	< 0.005	< 0.005
Liver,						
g/animal	9.37 ± 0.41	6.49 ± 0.08	12.15 ± 0.21	8.13 ± 0.32	< 0.005	< 0.005
g/100 g body wt	3.50 ± 0.08 (10)	3.79 ± 0.09 (10)	3.73 ± 0.05 (10)	4.01 ± 0.12 (10)	< 0.025	< 0.005
Spleen,						
g/animal	0.61 ± 0.015	0.45 ± 0.013	0.77 ± 0.028	0.53 ± 0.021	< 0.005	< 0.005
g/100 g body wt	0.23 ± 0.007 (10)	0.26 ± 0.008 (10)	0.24 ± 0.007 (10)	0.26 ± 0.009 (10)	NS [6]	0.065
Kidney,						
g/animal	1.54 ± 0.04	1.25 ± 0.02	2.05 ± 0.04	1.40 ± 0.06	< 0.005	< 0.005
g/100 g body wt	0.58 ± 0.029 (10)	0.73 ± 0.022 (10)	0.63 ± 0.013 (10)	0.70 ± 0.032 (10)	NS	< 0.005

[1] Statistical significance of germfree vs. conventional.
[2] Statistical significance of germfree and conventional males vs. germfree and conventional females.
[3] Body weight corrected for weight of cecal contents.
[4] Averages ± SE of mean.
[5] Number of rats.
[6] Not significant, $P > 0.05$.

between germfree and conventional rats, whereas the liver weight in germfree rats was slightly lower.

Plasma iron and copper, hemoglobin and hematocrit values are recorded in table 2. There was no significant sex difference or status difference (germfree vs. conventional) in hemoglobin and hematocrit values. Plasma iron and copper levels in the germfree rats were slightly but significantly lower than in the conventional animals. There was no sex difference in plasma copper values, whereas plasma iron values were substantially lower in males than in females.

The iron content of liver, spleen, kidney, and total body is summarized in table 3. The iron content of the liver, calculated both on 100-g fresh tissue basis (concentration) and on a total organ basis, was higher in germfree rats than in conventional rats. Germfree conditions decreased both the concentration and total iron in spleen, kidney, and total body. The concentrations of iron in liver, spleen, kidney, and total body, and the iron content of liver and total body were lower in male rats than in female rats.

Table 4 presents the mean values found for heme iron, storage (non-heme) iron, and the ferritin (water soluble) and hemosiderin (water insoluble) iron fractions of storage iron in the liver, spleen and kidneys of germfree and conventional female rats. The sum of the ferritin and hemosiderin iron fractions was almost equal to the storage iron in liver, spleen and kidneys. This indicates that all storage iron in these organs was accounted for by these procedures. The amount of hemoglobin iron present was subject to considerable variation even within the same organs, the percentage of heme iron in liver, spleen and kidney ranging from 7 to 12% in both conventional and germfree rats. This indicates that, under the experimental conditions, only a small portion of total tissue iron was present in the form of heme iron. The concentration of storage iron showed the same trend between germfree and conventional tissues as the total tissue iron (table 3). The concentration of both ferritin and hemosiderin iron was higher in liver and lower in spleen and kidneys of germfree rats, although the difference in

TABLE 2

Plasma iron and copper, blood hemoglobin, and hematocrit values in adult germfree and conventional rats [1]

	Germfree		Conventional		Significance (*P* values)	
	Male	Female	Male	Female	Status [2]	Sex [3]
Hemoglobin, g/100 ml	14.5 ± 0.16 [4]	14.3 ± 0.16	14.5 ± 0.15	14.7 ± 0.17	NS [5]	NS
Hematocrit, %	43.2 ± 0.34	43.1 ± 0.15	43.5 ± 0.33	43.4 ± 0.30	NS	NS
Plasma iron, μg/100 ml	145 ± 1.4	283 ± 4.4	154 ± 1.7	293 ± 2.7	< 0.005	< 0.005
Plasma copper, μg/100 ml	134 ± 1.3	136 ± 1.4	141 ± 1.2	142 ± 1.4	< 0.005	NS

[1] Ten rats/group.
[2] Statistical significance of germfree vs. conventional.
[3] Statistical significance of germfree and conventional males vs. germfree and conventional females.
[4] Averages ± SE of mean.
[5] Not significant, $P > 0.05$.

TABLE 3

Iron content of tissues and total body of adult germfree and conventional rats [1]

	Germfree		Conventional		Significance (*P* values)	
	Male	Female	Male	Female	Status [2]	Sex [3]
Liver,						
mg/100 g fresh tissue	21.7 ± 1.27 [4]	66.2 ± 1.46	16.8 ± 0.32	41.7 ± 1.63	< 0.005	< 0.005
mg/liver	2.21 ± 0.10	4.29 ± 0.08	2.04 ± 0.05	3.36 ± 0.10	< 0.005	< 0.005
Spleen,						
mg/100 g fresh tissue	51.7 ± 2.90	79.8 ± 3.19	162 ± 4.2	240 ± 12.6	< 0.005	< 0.005
mg/spleen	0.32 ± 0.018	0.37 ± 0.009	1.25 ± 0.03	1.20 ± 0.04	< 0.005	NS [5]
Kidney,						
mg/100 g fresh tissue	8.14 ± 0.17	9.30 ± 0.21	10.5 ± 0.35	15.3 ± 0.50	< 0.005	< 0.005
mg/kidney	0.13 ± 0.002	0.12 ± 0.003	0.22 ± 0.007	0.22 ± 0.011	< 0.005	NS
Total body,						
mg/100 g fresh wt	3.20 ± 0.09	6.47 ± 0.10	4.37 ± 0.06	8.15 ± 0.12	< 0.005	< 0.005
mg/total body	8.52 ± 0.20	10.4 ± 0.16	12.5 ± 0.27	15.6 ± 0.32	< 0.005	< 0.005

[1] Ten rats/group.
[2] Statistical significance of germfree vs. conventional.
[3] Statistical significance of germfree and conventional males vs. germfree and conventional females.
[4] Averages ± SE of mean.
[5] Not significant, $P > 0.050$.

TABLE 4

Heme, ferritin, and hemosiderin iron fractions of total tissue iron in liver, spleen, and kidney of adult germfree and conventional female rats [1]

	Liver		Spleen		Kidney	
	Germfree	Conventional	Germfree	Conventional	Germfree	Conventional
Heme iron,						
μg/g fresh tissue	57.2 ± 2.13 [2]	28.6 ± 1.22 [3]	70.1 ± 1.90	207 ± 5.3 [3]	7.32 ± 0.50	18.9 ± 1.32 [3]
% of total tissue iron	8.16 ± 0.36	7.08 ± 0.38	8.29 ± 0.14	8.91 ± 0.44	6.69 ± 0.95	12.5 ± 0.81 [4]
Storage (non-heme) iron,						
μg/g fresh tissue	650 ± 26	375 ± 20 [3]	768 ± 22	2090 ± 80 [3]	93.9 ± 2.7	139 ± 3 [3]
Ferritin iron,						
μg/g fresh tissue	439 ± 43	278 ± 13 [3]	386 ± 11	959 ± 45 [3]	76.0 ± 2.2	116 ± 1.5 [3]
% of storage iron	70.1 ± 0.41	74.2 ± 1.37 [4]	50.3 ± 0.29	45.9 ± 0.71 [3]	81.0 ± 0.75	84.0 ± 2.1
Hemosiderin iron,						
μg/g fresh tissue	192 ± 7	96.0 ± 6.5 [3]	385 ± 12	1122 ± 25 [3]	18.6 ± 0.91	22.0 ± 2.09
% of storage iron	29.6 ± 0.47	25.5 ± 1.01 [5]	50.1 ± 0.26	53.7 ± 1.33 [4]	19.8 ± 0.71	15.8 ± 1.43 [4]

[1] Six animals/group.
[2] Averages ± SE of mean.
[3] Difference between germfree and conventional rats significant at $P < 0.001$.
[4] Difference between germfree and conventional rats significant at $P < 0.05$.
[5] Difference between germfree and conventional rats significant at $P < 0.01$.

concentration of kidney hemosiderin iron did not reach statistical significance. There were only small though significant differences between germfree and conventional rats in the proportions of ferritin and hemosiderin iron. The ratio of hemosiderin iron to ferritin iron was higher in germfree liver and kidney than in the conventional organs but the ratio was lower in germfree spleen than in conventional spleen.

The concentration and content of copper in liver, spleen, kidney and total body are shown in table 5. The concentration of copper in these tissues varied between germfree and conventional rats, the general trend being similar to that of iron (table 3). Thus the concentration of copper in the liver was higher in germfree than in conventional rats. When these results were calculated on total liver content basis, there was no significant difference but even on this basis the germfree rat with generally lower liver weights tended to show a higher copper content. Germfree rats showed lower levels of copper in both spleen and kidney than the conventional rats. Total body copper was higher in the conventional rats than in the germfree. In both groups the values tended to be higher in females when expressed on a concentration basis, but higher in males when calculated on a total organ or a total body basis.

The concentration and content of manganese in the liver, spleen, kidney and total body of germfree and conventional rats are shown in table 6. Manganese was present in the least amount of the trace minerals measured in this investigation. There was no difference either between germfree and conventional rats or between sexes in the manganese concentration of liver, spleen and kidney. The concentration of manganese in the total body was slightly but significantly higher in females than in males, although the males being larger, had more total body manganese than the females. Whatever differences in total manganese content of body and organs were demonstrated, could be attributed entirely to the differences in organ weight or in body weight between the germfree and conventional rats and between the male and female rats.

TABLE 5

Copper content of tissues and total body of adult germfree and conventional rats [1]

	Germfree		Conventional		Significance (*P* values)	
	Male	Female	Male	Female	Status [2]	Sex [3]
Liver,						
μg/100 g fresh tissue	1094 ± 35 [4]	1374 ± 84	832 ± 28	891 ± 44	< 0.005	< 0.005
μg/liver	102 ± 4.9	88 ± 6.49	101 ± 3.4	73 ± 4.3	NS [5]	< 0.005
Spleen,						
μg/100 g fresh tissue	493 ± 24	516 ± 27	700 ± 16	743 ± 34	< 0.005	NS
μg/spleen	3.01 ± 0.15	2.41 ± 0.12	5.34 ± 0.43	3.89 ± 0.15	< 0.005	< 0.005
Kidney,						
μg/100 g fresh tissue	986 ± 52	1147 ± 43	1491 ± 48	1767 ± 70	< 0.005	< 0.005
μg/kidney	15.1 ± 0.66	14.4 ± 0.66	30.5 ± 1.21	24.7 ± 1.25	< 0.005	< 0.005
Total body,						
μg/100 g fresh wt	194 ± 5	223 ± 7	270 ± 6	295 ± 12	< 0.005	< 0.005
μg/total body	501 ± 8	360 ± 8	762 ± 22	552 ± 17	< 0.005	< 0.005

[1] Ten rats/group.
[2] Statistical significance of germfree vs. conventional.
[3] Statistical significance of germfree and conventional males vs. germfree and conventional females.
[4] Averages ± SE of mean.
[5] Not significant, $P > 0.05$.

TABLE 6

Manganese content of tissues and total body of adult germfree and conventional rats [1]

	Germfree		Conventional		Significance (*P* values)	
	Male	Female	Male	Female	Status [2]	Sex [3]
Liver,						
μg/100 g fresh tissue	237 ± 11 [4]	239 ± 9	227 ± 8	242 ± 12	NS [5]	NS
μg/liver	22.3 ± 1.40	15.2 ± 0.81	27.4 ± 0.88	19.7 ± 0.93	< 0.005	< 0.005
Spleen,						
μg/100 g fresh tissue	34.3 ± 2.86	28.8 ± 1.43	32.8 ± 1.36	35.9 ± 2.33	NS	NS
μg/spleen	0.21 ± 0.016	0.13 ± 0.006	0.26 ± 0.017	0.19 ± 0.012	< 0.005	< 0.005
Kidney,						
μg/100 g fresh tissue	117 ± 5	107 ± 2	109 ± 3	128 ± 8	NS	NS
μg/kidney	1.80 ± 0.09	1.34 ± 0.03	2.23 ± 0.08	1.79 ± 0.11	< 0.005	< 0.005
Total body,						
μg/100 g fresh wt	90 ± 4	108 ± 4	88 ± 4	98 ± 3	NS	< 0.005
μg/total body	230 ± 7	175 ± 7	248 ± 10	184 ± 6	NS	< 0.005

[1] Ten rats/group.
[2] Statistical significance of germfree vs. conventional.
[3] Statistical significance of germfree and conventional males vs. germfree and conventional females.
[4] Averages ± SE of mean.
[5] Not significant, $P > 0.05$.

DISCUSSION

The levels of iron, copper and manganese in diet L-462 are relatively high compared with requirements of rats under normal conditions. Early work in this laboratory had raised the question of the availability of trace minerals in steam-sterilized diets. This problem was emphasized more recently by the observation that the rabbits maintained with similar steam-sterilized diets demonstrated bone fragility, hind leg paralysis, irregular pregnancies, and low hemoglobin and plasma iron levels.[3] Availability of trace minerals after steam sterilization of diets was not known. Furthermore, variable losses could occur as a result of leaching of these trace minerals through the cloth bag in which the diet was sterilized. In view of these considerations, diet L-462, like most diets used for germfree animals, was fortified with relatively high levels of trace minerals. The general nutritional adequacy of this diet for germfree rats has been established through many generations of animals by its ability to support normal growth, performance and reproduction. No symptoms of toxicity of any trace minerals were observed in either germfree or conventional rats maintained with this diet.

It was shown previously that, when rats were fed a standard diet, there was no consistent difference in hemoglobin levels between males and females (13), but the plasma iron concentration was significantly higher in female rats (13, 14). The absence of any sex difference in plasma copper was also reported (1). The present work confirms these observations and indicates similar relationships in germfree rats. The observation of normal hemoglobin, hematocrit and plasma iron and copper levels in the germfree rats indicated the absence of any symptoms of anemia, in contrast with the anemic conditions observed in germfree rabbits maintained with a diet which produced totally asymptomatic conventional animals. Although the plasma iron and copper concentration in germfree rats can be considered to fall within the normal range, the values were slightly but significantly lower than in conventional animals.

Higher levels of storage iron in female rats were demonstrated by Kaldor and Powell (15) and Morgan (14). The present data are in agreement with these conclusions (14, 15) and indicate the existence of a similar situation in germfree rats. Data on the influence of the sex factor on the copper and manganese content of organs and of total body are sparse. Spray and Widdowson (16) observed no striking sex difference in the copper content of the total body, although female rats tended to have greater concentrations than males of the same ages. The present data show that the concentrations of copper in liver, kidney and total body of female rats are higher, although the greater organ and total body weights of male rats resulted in higher total content of this mineral. The results also indicate that the over-all effect of sex observed in the case of copper was small compared with that of iron. In the case of manganese there was no difference in the specific organs tested, but an effect of sex on total body concentration and content comparable to that of copper could be demonstrated.

The liver of the germfree rat stored more iron than the liver of the conventional animal, but the spleen and kidneys contained less iron, although all animals were fed the same sterilized diet. From this fact it could be postulated that the spleen might not be a major iron storage organ in germfree animals. Kaldor (17) reported that, in the absence of the spleen, the liver appeared to assume the storage function of this organ because the difference between the iron content of the livers of similarly treated intact rats and splenectomized rats was approximately equal to the amount of iron in the spleens of intact rats. Since the combined liver and spleen storage iron was not appreciably different between germfree and conventional animals, it is possible that the changes in iron content noted in the liver and spleen of germfree rats represent a shift in function between these 2 organs. Moreover, recent studies (18) have shown that germfree rats contained approximately 22% less total blood volume and

[3] Reddy, B. S., J. R. Pleasants and B. S. Wostmann 1964 Iron and copper metabolism in germfree rabbits and rats. Gnotobiotic Symposium and Workshop, Michigan State University, East Lansing, Michigan.

hemoglobin per 100 g body weight than conventional rats. This suggests that in germfree rats less iron is needed for hemoglobin formation. Mazur and Carleton (19) stated that, in endotoxin-treated rats, the low amount of iron available to bone marrow for hemoglobin synthesis was associated with an abnormal accumulation of iron in the liver. In the germfree rats a lower demand for iron for hemoglobin formation would then be associated with an abnormal accumulation of storage iron in the liver, whereas the lower iron levels noted in the spleen would reflect a generally reduced iron metabolism.

The occurrence of hypochromic anemia in germfree rabbits focussed attention on the availability and absorption of dietary iron in germfree animals. It has been demonstrated that alterations of the intestinal flora by antibiotic treatment were associated with a decrease in iron absorption (20, 21). A recent investigation showed a more positive oxidative-reduction potential in the intestine of the germfree rat as compared with the conventional rat, the difference being of the order of 200 to 300 mv.[4] These data suggest that the availability of iron could differ between germfree and conventional animals, depending on its ionic state and the amount of iron-complexing material present in the diet (22). In germfree rabbits, the symptoms of hypochromic anemia were, presumably, due to insufficient availability of iron from the added ferric citrate under the conditions existing in the germfree intestine. Changes in the diet composition which provided less total iron in a more natural form (soybean meal) apparently relieved symptoms of anemia.[5] It is probable that in germfree rats also, the iron availability may be slightly lower due to existing intestinal conditions. However, the absorption of iron is sufficient to meet requirements for hemoglobin formation and even allows a higher than normal storage of iron in liver.

The results of the separation of storage iron in conventional female rats are consistent with previous reports which indicated that, at approximately 4 months of age, ferritin storage predominated in the liver and hemosiderin storage predominated in the spleen (2). No comparable data were available in the literature for the rat kidney. Although a considerable difference was observed between germfree and conventional rats with respect to storage iron content of liver, spleen and kidney, the relative proportions of the ferritin and hemosiderin iron fractions in these organs were approximately constant in germfree and conventional rats. In general, these data are in agreement with the observations that the iron appears to be stored in and to be released equally readily from the ferritin and hemosiderin compounds in normal rats (23). The present data suggest that the germfree rats resemble conventional rats in this respect.

The levels of copper in liver, spleen, kidney and total body followed the same pattern as the iron levels in germfree and conventional rats, as demonstrated by an increase in concentration of liver copper and decrease in spleen, kidney and total body copper levels in germfree rats. Although copper concentrations were much lower than concentrations in the comparable iron data, similar ratios between the values obtained from germfree and conventional animals generally existed. Thus the apparent iron-copper relationship holds true in germfree rats as well as in conventional rats and thereby supports the general conception of the relation of copper to iron metabolism in the animal body. The levels of copper observed in the livers of both germfree and conventional rats are somewhat higher than reported by others (24). This may reflect the relatively high copper concentration of diet L-462 mentioned previously.

The manganese data presented for conventional rats are in agreement with the observations of Lorenzen and Smith (24) for the liver of rats, and with the average figures on liver, spleen and kidney recorded in the literature for a wide range of species (25). In contrast with the observations on iron and copper there was no significant difference between germfree and conventional rats in the concentrations of manganese in liver, spleen and kidney. The relative constancy of the manganese levels indicates that the germfree state did not appreciably change the

[4] Unpublished data, B. S. Wostmann, 1964.
[5] See footnote 3.

over-all status of this mineral in the animal body. The fact that no influence of the sex factor was observed on the manganese levels in spleen, liver and kidney, whereas both germfree and conventional females demonstrated a somewhat higher total body manganese concentration, suggests that in females the manganese concentration of the bones must be higher, since a major part of the total manganese is situated in the skeleton (1).

Because of the knowledge obtained thus far, it appears reasonable to state that in germfree rabbits, normal hemoglobin levels were obtained only by providing iron in a form whose availability was not seriously affected by germfree intestinal conditions. The present data on rats suggest a lower rate of metabolism of iron and copper in germfree rats than in conventional rats and an inappreciable difference in manganese metabolism between germfree and conventional rats. Even though iron may conceivably be less available in the intestine of the germfree rat, the absorption and utilization are sufficient to provide normal hemoglobin levels for a reduced total volume. In fact, the metabolism of iron in germfree rats results in even higher levels of liver iron than observed in conventional rats.

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Zinc-65 Metabolism during Low and High Calcium Intake in Man [1]

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ABSTRACT The metabolism of orally administered tracer doses of Zn^{65} was studied in man under strictly controlled dietary conditions during low and high calcium intake. The average intestinal absorption of radiozinc was 35.7% in the 5 patients studied during low calcium intake. There was no significant change in Zn^{65} absorption when the intake of calcium of these patients was increased six- to tenfold. Also, there was no correlation between the calcium balances and the absorption of Zn^{65}, nor between the urinary excretion of calcium and of Zn^{65}. A calcium-zinc antagonism, postulated in animal experiments, could not be demonstrated in humans under the conditions of our studies.

The interaction of zinc and calcium has been extensively described (1–5), as well as the influence of trace elements on the skeleton (1, 6). The addition of large amounts of calcium to the diet of animals, receiving a borderline intake of zinc, has been shown to induce symptoms of zinc deficiency (1, 2, 7, 8).

Only limited information is available on the metabolism of stable zinc in man, probably because of the problems of trace metal contamination inherent in studies of trace metal metabolism. The use of radioactive zinc (Zn^{65}) has eliminated some of these difficulties, and has facilitated studies of some aspects of zinc metabolism in man. The disappearance rate of Zn^{65} from the plasma and its uptake in white and red blood cells have been investigated (9, 10), and the tissue distribution and the turnover of Zn^{65} have been determined in man (11). To our knowledge, no information is available on the absorption of Zn^{65} from the intestine under controlled dietary conditions in man, or on the influence of different dietary factors on Zn^{65} absorption in humans. Studies of the absorption of zinc were therefore performed in man under constant conditions using Zn^{65} as the tracer and on the effect of calcium added to the diet.

EXPERIMENTAL

The studies were performed under strictly controlled dietary and metabolic conditions in our metabolic research ward on 5 patients during low and high calcium intake (table 1). All patients were ambulatory and in good physical and nutritional state. The only objective observations were the residue of a previous dermatitis in patient 1, some spurring of the vertebrae on X-ray in patient 2, the slight-to-moderate demineralization of the skeleton in patients 3 and 5, and the pulmonary calcifications on X-ray of the chest in patient 4. All chemical laboratory tests of these patients were normal.

The diet was kept constant throughout the studies and was analyzed for nitrogen, calcium, phosphorus, sodium and potassium. The composition of the diet is listed in table 2. The dietary content of stable zinc was calculated to range from 12 to 15 mg/day. The daily fluid intake was kept constant. The body weight and the urine volume were determined daily. All urine and stool specimens were collected from the start of the study. Metabolic balances of calcium, phosphorus and nitrogen were determined during the low and high calcium intake to ascertain the metabolic status of the patients. Five studies of the absorption of Zn^{65} were performed during the intake of a low calcium diet which contained an average of 258 mg calcium and 771 mg phosphorus/day. Subsequently, 8 absorption studies of Zn^{65} were performed in the same patients dur-

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TABLE 1

Patients studied and duration of studies

Patient [1] no.	Age	Diagnosis	No. of days studied	
			Low calcium diet [2]	High calcium diet [3]
	years			
1	50	Chronic dermatitis	24	30
2	51	Osteoarthritis	28	14, 21
3	60	Osteoporosis, moderate	22	22
4	60	Pulmonary calcifications and emphysema	20	14, 15
5	51	Osteoporosis, mild	24	22, 10

[1] All subjects were male patients.
[2] Average low calcium intake = 253 mg/day.
[3] Average high calcium intake = 1981 mg/day.

TABLE 2

Composition of metabolic low calcium diet (2223 kcal)

	Protein	Carbohydrate	Fat
	g/day	*g/day*	*g/day*
Fruit juice, 200 ml		24	
Rice,[1] 30 g	22	24	
Spaghetti,[1] 20 g		15	
Bread, 150 g		78	
Jelly, 120 g		78	
Potatoes,[2] 100 g	5	19	
Canned vegetables, 175 g		15	
Sweetened canned fruit, 225 g		44	
Sugar, 28 g		28	
Meat (beef and turkey), 200 g	44		28
Butterfat, 50 g			41
Cream, 20 g			2
Total	71	325	71

[1] Weight of raw food.
[2] Weight of cooked food.

ing high calcium intake. Two studies were performed during high calcium intake in patients 2, 4 and 5. An interval of 3 to 10 months was allowed to elapse before the studies were performed during high calcium intake in order to permit the disappearance of Zn^{65} from the blood. In the high calcium phase, calcium gluconate tablets were added to the constant low calcium diet to raise the calcium intake approximately eightfold, all other constituents of the diet remaining unchanged; the high calcium intake ranged from 1707 to 2123 mg/day and averaged 1983 mg/day.

A single tracer dose of 15 to 25 μc Zn^{65} as the chloride was administered orally with breakfast during low and high calcium intake. The levels of Zn^{65} in whole blood and plasma were determined at 1, 4, 8 and 24 hours on the day of administration of the Zn^{65} tracer, daily in the first week of the study and at less frequent intervals thereafter.

The urinary Zn^{65} excretions were determined daily on aliquots of 24-hour urine collections and the fecal Zn^{65} excretions were determined on each stool specimen. The duration of the tracer studies averaged 24 days during low calcium intake and 19 days during high calcium intake.

The counting of Zn^{65} in plasma, whole blood, urine and stool was performed in a well-type NaI crystal γ-scintillation counter. Dose standards were counted at the time of the radioassays of the samples of blood, urine, and stool. The Zn^{65} level in whole blood and plasma was expressed as percentage of dose per liter, the urinary Zn^{65} excretion as percentage of dose per total urine volume per day, and the fecal Zn^{65} excretion as percentage of the administered dose per stool specimen.

The absorption of Zn^{65} was determined as follows:

$$\text{Absorption, \%} = \frac{\text{Zn}^{65}\text{ intake} - \text{fecal Zn}^{65}}{\text{Zn}^{65}\text{ intake}} \times 100.$$

In this calculation, the endogenous fecal excretion of Zn^{65} has not been considered. The true absorption of Zn^{65} was determined by correcting for endogenous fecal Zn^{65} ex-

cretion following the intravenous injection of the tracer to man (11), considering an average of 10% of the absorbed dose as endogenous fecal Zn^{65} excretion.

The urinary excretions of creatinine, calcium and phosphorus were determined daily. Metabolic balances of calcium, phosphorus and nitrogen were determined on aliquots of 6-day pools of urine and stool and on aliquots of the diet. Calcium was determined by precipitation as the oxalate and titration with permanganate; nitrogen by the Kjeldahl method, and phosphorus by the method of Fiske and Subba-Row (12). Stool calcium and phosphorus were determined on dry-ashed aliquots of 6-day stool pools.

RESULTS

A representative example of the Zn^{65} levels in whole blood and plasma in the first 24 hours following the ingestion of a single tracer dose of Zn^{65} is shown in figure 1 (patient 1). Initially, the Zn^{65} level in plasma was higher than that of whole blood, reached a maximum at 4 hours and decreased with time thereafter. The Zn^{65} level in whole blood reached a maximum also at 4 hours; however, this level decreased only slightly by the eighth hour, and increased thereafter so that the Zn^{65} level in whole blood was approximately twice as high at 24 hours as at one hour after the ingestion of the dose.

The Zn^{65} level in plasma and whole blood in 28 days after the oral ingestion of Zn^{65} is shown in figure 2. The plasma concentration of Zn^{65} continued to decrease with time and less than 0.1% of the dose remained per liter of plasma at 10 days. From day 20 to 28 after the oral administration of the dose, the plasma level of Zn^{65} remained approximately in the same low range. In contrast, the Zn^{65} level in whole blood continued to increase in the first 5 days after the ingestion of the tracer, and decreased thereafter. However, the Zn^{65} level in whole blood remained about 2 to 3 times as high as the plasma level throughout the 28 days of the study.

The urinary and fecal excretion of Zn^{65} after the oral administration of the dose is graphed in figure 3 (patient 2). The urinary excretion of Zn^{65} was very low throughout the study and was somewhat higher in the first few days after the administration of Zn^{65} than in the later phase of the study when less than 0.1% of the dose was excreted in urine per day. The major portion of the administered dose, 66%, was passed with the stool in the first 3 days (not graphed in fig. 3). Thereafter, the fecal excretion of Zn^{65} was low and less than 1% of the dose was excreted per

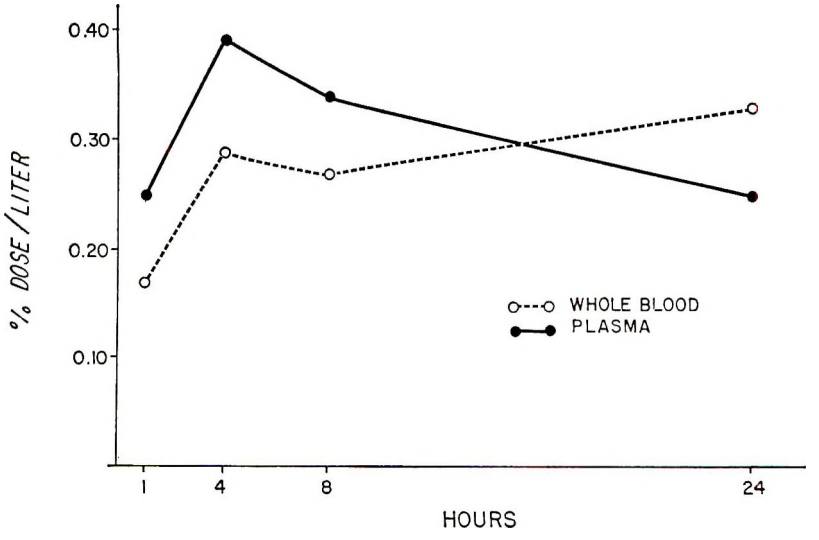

Fig. 1 Zn^{65} levels in plasma and whole blood in man; a single tracer dose of $Zn^{65}Cl_2$ was given orally at the start of the study.

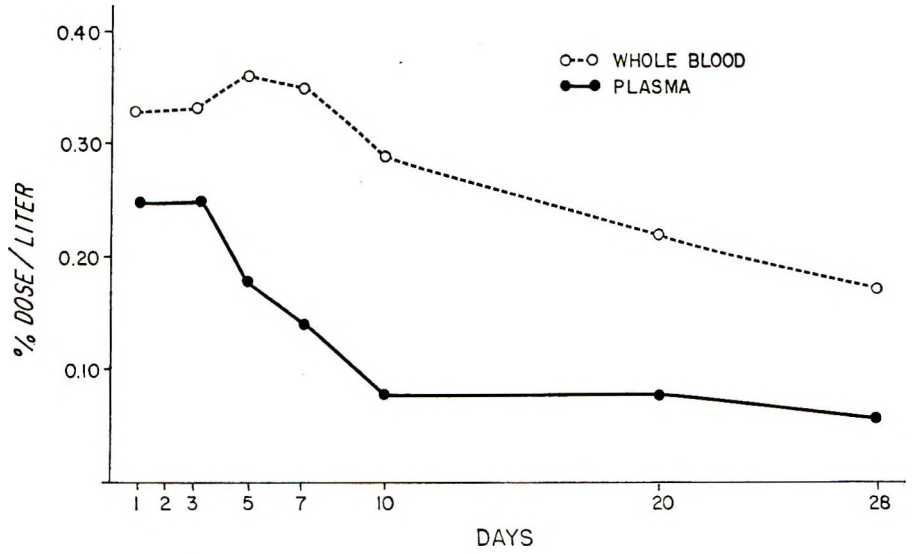

Fig. 2 Zn^{65} levels in plasma and whole blood in man during low calcium intake; a single tracer dose of $Zn^{65}Cl_2$ was given orally at the start of the study.

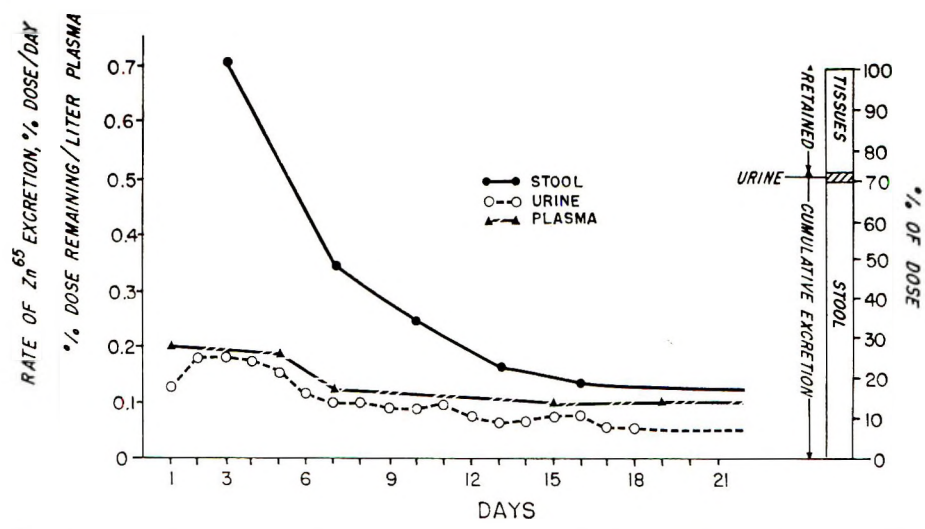

Fig. 3 Metabolism of an oral tracer of Zn^{65}; plasma levels, rate of excretions, cumulative excretions and tissue retention of Zn^{65}; a total of 66% of the orally administered Zn^{65} was passed with the stool in the first 3 days.

day. During the later phase of the study, the excretion of Zn^{65} in stool was approximately 0.2% of the dose per day and this excretion was slightly higher than the urinary Zn^{65} excretion. At the end of 21 days the major portion of the ingested Zn^{65}, 70%, had passed with the stool, 2% in urine, and 28% of the dose was retained in the tissues of the body.

The excretion pattern of Zn^{65} in urine and stool in the remaining 4 patients was similar to that of patient 2.

The Zn^{65} levels in plasma and whole blood during low and high calcium intake are shown in figure 4 (patient 2). During both intake levels of calcium, the plasma level of Zn^{65} reached a peak at 4 hours. During high calcium intake, the Zn^{65} levels

in plasma and in whole blood were slightly lower at 8 hours and at 24 hours than at the corresponding time intervals during low calcium intake. This pattern of Zn^{65} concentration in plasma and whole blood during high calcium intake was noted in all but one of the 5 patients studied (patient 1), in whom the Zn^{65} level in whole blood was higher during high calcium intake than during low calcium intake.

Since the passage time of the radioisotope in stool varies greatly in different persons, the fecal Zn^{65} excretion graphed in figure 5 represents the cumulative Zn^{65} excretion up to the time when less than 1% of the dose was excreted in stool per day. During low calcium intake the cumulative fecal Zn^{65} excretion ranged from 61.5 to 75.7% in the 5 patients. The effect of high calcium intake was neither consistent nor

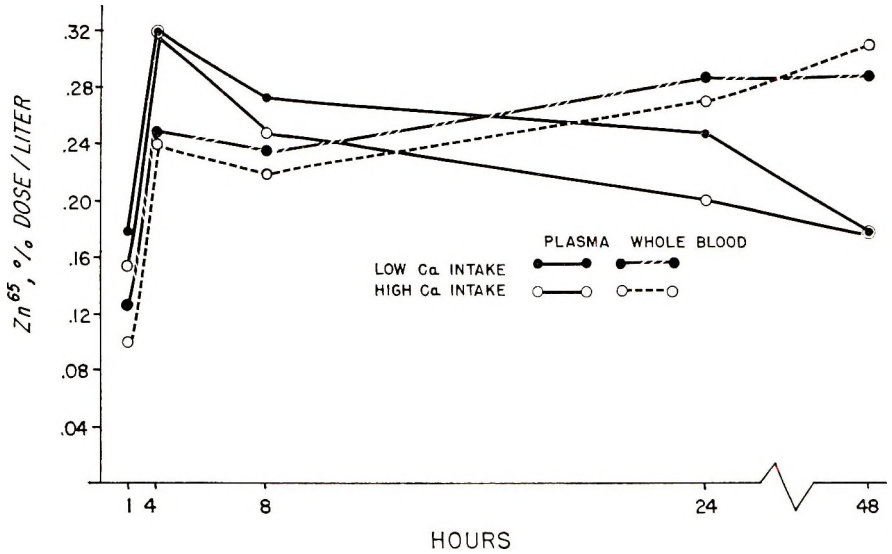

Fig. 4 Concentration of Zn^{65} in whole blood and plasma during low and high calcium intake; a single tracer dose of $Zn^{65}Cl_2$ was given orally at the start of the study.

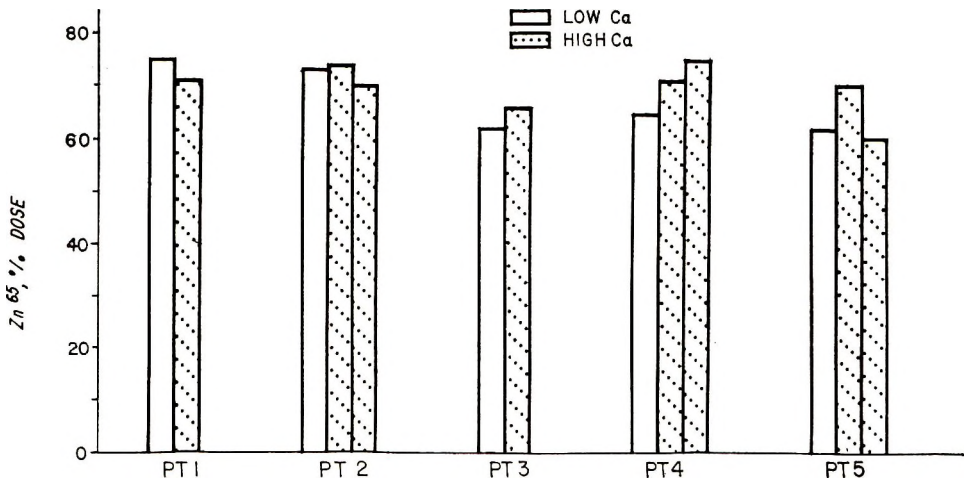

Fig. 5 Fecal excretion of Zn^{65} during low and high calcium intake; each bar represents the cumulative fecal Zn^{65} excretion up to the time when less than 1% of the dose was excreted in stool.

significant and the fecal Zn^{65} excretion was approximately in the same range during low and high calcium intake in patients 1, 2 and 3 and was slightly higher during high calcium intake in patients 4 and 5. The Zn^{65} excretion in stool averaged 72.7% in the 8 studies performed during high calcium intake as compared with an average excretion of 68.1% during low calcium studies.

Table 3 shows the relationship between the calcium balances and Zn^{65} absorption during low and high calcium intake. The calcium balances were slightly negative with low calcium intake and became positive on addition of calcium to the diet. There was no correlation between the calcium balances and the intestinal absorption of Zn^{65} (net or corrected for endogenous fecal Zn^{65}) with low or high calcium

TABLE 3

Relationship between calcium balances and Zn^{65} absorption in man

Patient no.	Low calcium intake				High calcium intake			
	Calcium		Zn^{65} absorption		Calcium		Zn^{65} absorption	
	Intake	Balance	Apparent [1]	True [2]	Intake	Balance	Apparent [1]	True [2]
	mg/day	*mg/day*	*% dose*	*% dose*	*mg/day*	*mg/day*	*% dose*	*% dose*
1	260	− 94	24.3	27.0	2123	+359	26.5	29.4
2	247	−144	27.2	30.2	2048	+ 62	24.7	27.4
					1974	+101	29.4	32.7
3	174	− 38	39.5	43.9	1707	+301	30.9	34.3
4	304	−161	33.7	37.5	2017	+ 29	27.3	30.5
					1864	+126	22.5	25.0
5	305	−104	35.9	39.9	2022	+220	34.7	38.9
					2105	+390	28.8	31.9
Average	258	−108	32.1	35.7	1983	+199	28.1	31.3

[1] Apparent absorption, $\% = \frac{Zn^{65}\text{ intake} - \text{fecal } Zn^{65}}{Zn^{65}\text{ intake}} \times 100$. Not corrected for endogenous fecal Zn^{65} excretion.

[2] Corrected for endogenous fecal Zn^{65} excretion.

TABLE 4

Excretion of calcium and of Zn^{65} in urine

Patient no.	Calcium intake	Urinary Zn^{65} excretion		Urinary calcium excretion
		24-hour	7-day cumulative	
	mg/day	*% of dose*	*% of dose*	*mg/day*
1	242	0.10	0.55	74
	2084	0.20	1.12	76
2	240	0.10	0.25 [1]	65
	2020	0.06	0.38 [1]	88
	1944	0.11	0.80	86
3	168	0.11	0.90	71
	1713	0.13	1.03	187
4	314	0.13	0.82	122
	1881	0.11	0.63	161
	1994	0.12	0.74	206
5	300	0.19	1.07	71
	2093	0.01	0.65	48
	2115	0.08	0.66	46

[1] Four-day cumulative excretion.

intake nor was there any major change in the Zn^{65} absorption in the high calcium phase. The corrected Zn^{65} absorption averaged 35.7% during low calcium intake and 31.3% during high calcium intake.

Table 4 shows the relationship between the urinary excretion of Zn^{65} and of calcium during low and high calcium intake. There was no consistent trend of change in urinary Zn^{65} excretion on addition of calcium to the diet, the urinary Zn^{65} excretion increasing in some studies and decreasing in others in the high calcium phase. This was the case for the Zn^{65} excretion in the first 24 hours after the administration of the dose, and for the 7-day cumulative urinary Zn^{65} excretion.

DISCUSSION

After the oral administration of a radioisotope, the plasma level reflects the rate and extent of entry of the tracer from the intestine into the blood stream. Since the Zn^{65} plasma level was highest 4 hours after the ingestion of the dose and decreased with time thereafter, it appears that most of the absorption of Zn^{65} from the intestine was accomplished during the first 4 hours. The subsequent continuous decrease in the Zn^{65} plasma level was probably due to the uptake of Zn^{65} by the various tissues and by cellular elements of the blood (9–11) and to binding of Zn^{65} to plasma proteins (13, 14). The high Zn^{65} level in whole blood in the 28-day study indicated continued incorporation of Zn^{65} into red and white blood cells, in agreement with observations made following the intravenous injection of Zn^{65} in man (9, 11).

The low urinary excretion of Zn^{65} after the oral ingestion of Zn^{65} is similar to observations in man following the intravenous administration of Zn^{65} (11). The main pathway of Zn^{65} excretion in man, after either oral or intravenous administration of the radioisotope, is via the intestine and not via the kidney, similar to observations made in rats (15). There was no correlation between the Zn^{65} plasma levels and the urinary excretion of Zn^{65} in man, in agreement with results obtained in mice (13).

The fecal Zn^{65} excretion varied in the 5 patients during low calcium intake and the major portion of the fecal Zn^{65} represented Zn^{65} which passed unabsorbed through the intestine. Because of the long physical half-life of Zn^{65} and of its long biological half-life in man (11), an intravenous injection of Zn^{65} could not be given to patients who had previously received an oral dose of this isotope. The endogenous fecal excretion of Zn^{65} could therefore not be measured in these patients. However, the endogenous excretion of Zn^{65} could be estimated from the information obtained in a different group of patients who had received this radioisotope intravenously (11). The fecal Zn^{65} excretion after an intravenous dose averaged 12.8% in 6 patients in 12 days, whereas the excretion in urine averaged 1.6%. On the basis of these results it was assumed that an average of 10% of the absorbed dose of Zn^{65} was secreted into the intestine in 12 days. When this figure was used for the calculation of the endogenous fecal Zn^{65} excretion, no significant difference was found in the true absorption of Zn^{65} from the intestine during low and high calcium intake (table 3).

The absorption of Zn^{65} in man, during either a low or a high calcium intake, was higher than the absorption of zinc in steers (16), rats (17) or mice (13). However, the studies in rats and steers were performed with diets containing relatively larger amounts of calcium and of stable zinc than given to humans. Recently, the absorption of Zn^{65} in rats, determined by total body counting, has been reported to be approximately 40% (15), which is similar to the values obtained in our studies in man.

The effect of added calcium on Zn^{65} absorption was variable in the present study and there was no consistent change in the absorption of Zn^{65} during high calcium intake. Also, there was no consistent relation between the urinary excretion of Zn^{65}, the intake of calcium and the increase in urinary calcium excretion in man. The diagnoses of patients 2 to 5 may indicate disturbances of calcium metabolism which may have influenced the absorption of Zn^{65}. However, as in normal persons receiving a low calcium intake, the calcium balances of these patients were only slightly negative with the low calcium diet and they became positive to

varying degrees on adding calcium to the diet, similar to observations made in persons who do not have these diagnoses.[2] Stable zinc has been shown to influence the absorption of Zn^{65} (18, 19). The zinc content of the diet in the present study was calculated and not analyzed and variations in zinc intake may therefore have occurred. However, it is unlikely that this factor may have obscured any lack of effect of added calcium on the absorption of Zn^{65} since the dietary intake was kept constant throughout the studies, the only change being the addition of calcium gluconate tablets during the high calcium study.

The tracer dose of Zn^{65} was used as the chloride and zinc in this form may differ from zinc contained in natural foods. As the dose of Zn^{65} was given at the midpoint of breakfast, it is likely that Zn^{65} did not remain in ionic form but mixed with food and may have been bound to proteins. Since Zn^{65} was given with a single meal, whereas zinc contained in the diet is ingested with each meal, it might be inferred that the results obtained in a single-dose study do not represent the metabolism of this element after repeated intake. No data are available on the comparative metabolism of single and multiple doses of Zn^{65} in man. However, studies of the metabolism of single and multiple doses of Sr^{85} and Ca^{45} in man have shown that the metabolism of the single dose is an indicator of the metabolism of multiple doses (20). If Zn^{65} metabolism follows a similar pattern, it may be assumed that the metabolism of the single dose of Zn^{65} reflects the metabolism of multiple doses and represents the metabolism of stable zinc. The absorption of Zn^{65} may also depend on the time of ingestion of the tracer in relation to the calcium intake with the meal, for instance, whether Zn^{65} is ingested with the first meal in the morning or several hours later, after the ingestion of several meals. This aspect has not been investigated in reference to Zn^{65} absorption in the present study. However, the relationship of the time of administration of another element, Ca^{47}, and of its absorption in man, has been studied in this laboratory and no significant difference in calcium absorption was noted when the tracer was given with the morning and evening meal during either a low or high calcium intake.[3]

The mechanism by which calcium affects zinc metabolism in animals has not been clearly defined (21). Several factors may play a role in the interaction of the 2 elements in the body, such as interference of calcium with the absorption of zinc from the intestine and its excretion in urine, the distribution of zinc in various tissues, the relation between loosely and tightly bound zinc in plasma (14), and the action of enzymes. Calcium added to the diet of experimental animals has been reported to decrease the absorption of zinc in some studies (22–25), and not to change zinc absorption in other studies (26). In a recent review on the antagonism between calcium and zinc in animals, it has been demonstrated that calcium interferes with the absorption of zinc from the intestine in rats (15). The retention of orally administered Zn^{65} decreased progressively as the amounts of calcium added to the diet were increased. The amount of calcium ingested by the rats, relative to the total body weight, was very much higher than the levels of calcium ingested in our studies in humans. Therefore, the lack of change in the absorption of Zn^{65} during low and high calcium intake in man may be due to the difference in the amounts of calcium used in the animal experiments and in man. Another reason for these differences may be the low phytic acid content of the human diet. It has been demonstrated in animals that phytic acid is necessary for the interference of calcium with the utilization of zinc (8, 27, 28).

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Fatty Acid Composition of Rat Liver Lipids during Choline Deficiency [1]

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ABSTRACT The fatty acid compositions of whole liver triglycerides, diglycerides, total phospholipids, and lecithin, and mitochondrial total phospholipids and lecithin were determined on rats which had developed fatty livers after they had been fed a low protein, choline-deficient diet for 10 or 28 days. Experiments were conducted with either a hydrogenated vegetable oil or safflower oil as the source of dietary fat. Overall, there was essentially no difference in the fatty acid patterns of the liver lipids from the choline-deficient animals when compared with those receiving a choline supplement. However, the type of fat and carbohydrate in the diet accounted for changes in the fatty acid composition in both the choline-deprived and choline-fed rats. The hydrogenated vegetable oil, which has a high amount of oleic acid, caused the liver lipid oleic acid content to increase and produced a lowering of the linoleic and arachadonic acid levels, whereas safflower oil, containing a high content of linoleic acid, caused the liver lipid oleic acid level to drop while the linoleic acid level remained nearly constant. An increase in the palmitic acid and palmitoleic acid content of all the liver lipids analyzed was observed and was attributed to the high carbohydrate content of the semi-purified diets. It was concluded that the accumulation of liver neutral lipid during choline deficiency in the rat is not accompanied by changes in the fatty acid patterns of liver neutral lipids or liver phospholipids which can be ascribed directly to the lack of choline. The alterations noted in the liver lipid fatty compositions may be attributed to changes in the dietary fat and to the dietary carbohydrate levels.

Since the discovery by Best and Huntsman in 1932 (1) that choline exhibited lipotropic properties, much effort has been spent in attempting to elucidate its mode of action. A fatty liver may arise as a result of increased hepatic lipid synthesis, reduced hepatic lipid degradation, stimulated transport of fat to the liver, decreased mobilization of fat from the liver, or a combination of these (2).

Studies on fatty acid synthesis in the livers of normal versus choline-deficient animals have been carried out by many investigators (3–5). Using D_2O it has been shown that livers of choline-deficient rats have more newly synthesized fatty acids than the normal rat liver (4). On the other hand, no difference was noted in the amount of newly synthesized hepatic fatty acids from C^{14}-acetate in choline-deficient and normal rats (5). Investigations have established that choline-deficient diets containing a high content of saturated fatty acids cause a greater accumulation of liver fat than diets rich in unsaturated fatty acids (6–8). Using choline-deficient diets, Stetten and Salcedo (9) demonstrated that the amount of fat accumulated in the liver increased as the chain length of the dietary fatty acids decreased from stearic acid to myristic acid.

It is well established that a dose of choline given to animals maintained with a choline-deficient diet stimulates the incorporation of P^{32} into liver phospholipids (10–13). Cornatzer and Gallo have also shown that the level of liver lecithin increases after the administration of a single dose of choline to rats rendered choline-deficient (14). More recently Cornatzer and Walser (15) demonstrated that a dietary choline-deficiency produces a decrease in liver lecithin and an increase in phosphatidic acid.

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Since fatty acids are structural components of phospholipid and neutral lipid molecules which undergo altered metabolism during choline deficiency, we thought it to be of benefit to study the fatty acid composition of various liver lipids of choline-deficient animals in order to gain further insight as to the lipotropic action of choline. Experiments in which fatty livers were produced by a choline-deficient, low protein diet will be reported. These experiments were designed to determine whether the fatty acid composition of the liver lipids synthesized during choline-deficiency was different from liver lipids of animals receiving the same diet supplemented with choline. Dietary fat was varied with respect to fatty acid composition by using either safflower oil[3] or a hydrogenated vegetable oil.[4] If the fatty acid patterns of liver lipids undergo changes during choline deficiency in may help to explain why neutral lipid accumulates in this abnormal physiological state.

EXPERIMENTAL

Male, Sprague-Dawley strain rats,[5] 73 days of age at the beginning of the dietary regimens, were used in the experiments and were housed in wire-bottom cages. The animals were maintained with laboratory chow [6] prior to the low protein (5%), low fat (5%) semi-purified diets. The gross composition of the prepared diets is shown in table 1 and is similar to that used previously by Cornatzer andi co-workers (13–16). Animals were maintained with these diets for 10 or 28 days and were allowed free access to food and water. Normal and experimental animals were decapitated at the prescribed days and the livers removed and divided into 2 approximately equal portions, one portion for the preparation of mitochondria by differential centrifugation (17) and the other used for the whole liver studies.

Total lipids were extracted from the liver mitochondria and whole liver with chloroform-methanol (2:1), and water-soluble contaminants were removed by the methods of Folch et al. (18). The whole liver total lipid extract was separated into neutral lipid and phospholipid fractions by a silicic acid column as described by Borgstrom (19). The neutral lipid fraction was further separated into cholesterol ester, triglyceride, cholesterol, diglyceride and monoglyceride fractions by the method of Carroll (20). The fatty acid composition was determined on only the triglyceride and diglyceride fractions. The isolation of lecithin from the phospholipid fraction was carried out with silicic acid-impregnated glass fiber paper chromatography as described by Cornatzer et al. (21).

TABLE 1

Composition of diet [1]

Casein [2]	5
Cod liver oil [2]	1
Hydrogenated vegetable oil [3] or safflower oil [4]	4
Sucrose	42
Dextrin	42
Non-nutritive bulk [2]	2
Salt mixture [5]	4
Vitamin mixture [6]	1
Choline chloride (when supplemented)	0.5

[1] Expressed as per cent by dry weight.
[2] Nutritional Biochemicals Corporation, Cleveland.
[3] Crisco, Procter and Gamble Company, Cincinnati.
[4] Saff-o-life, General Mills, Inc., Minneapolis.
[5] U.S.P. XIII, including: (per cent by weight) calcium biphosphate, 13.58; calcium lactate, 32.70; ferric citrate, 2.97; magnesium sulfate, 13.70; potassium phosphate (dibasic), 23.98; sodium biphosphate, 8.72; sodium chloride, 4.35 (Nutritional Biochemicals Corporation, Cleveland).
[6] Including: (per cent by dry weight) dextrin, 96.40; thiamine HCl, riboflavin and pyridoxine, 0.08 each; nicotinic acid, 2.40; Ca pantothenate, inositol and p-aminobenzoic acid, 0.32 each.

The total lipid extract of the mitochondria was concentrated and used for the determination of total lipid fatty acids and also for the isolation of lecithin by paper chromatography as described above.

Aliquots of the whole liver total, neutral, and phospholipid extracts and the mitochondrial total lipid extract were evaporated to dryness and the lipid content determined gravimetrically.

Methyl esters of the whole liver total phospholipids, lecithin, triglycerides, and diglycerides and mitochondrial total lipids and lecithin were prepared by the methods of Morgan et al.[7] The fatty acid methyl esters of the vegetable oils were obtained by refluxing the material with 5% H_2SO_4 in methanol (22). The lipids of the lab-

[3] Nutritional Biochemicals Corporation, Cleveland.
[4] Crisco, Procter and Gamble Company, Cincinnati.
[5] Sprague-Dawley, Incorporated, Madison, Wisconsin.
[6] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[7] Morgan, T. E., D. J. Hanahan and J. Eckholm 1963 A rapid method for the deacylation of phospholipids and neutral lipids. Federation Proc., 22: 414 (abstract).

oratory chow were extracted with chloroform-methanol (2:1) and the fatty acid methyl esters prepared in a manner similar to that used for the vegetable oils. The fatty acid composition of the laboratory chow and the oils used in the diets is shown in table 2. Oleic acid and linoleic acid, of the hydrogenated vegetable oil, include some geometric and positional isomers of these compounds which are formed during the hydrogenation process. These isomers of oleic and linoleic acids are not separated during chromatography and consequently all the isomers of oleic acid and of linoleic acid are included in table 2 under the designation 18:1 (oleic acid) and 18:2 (linoleic acid).

The fatty acid methyl esters were characterized by gas-liquid chromatography on a gas chromatograph [8] equipped with an argon ionization detector with an Sr^{90} radioactive source. The stationary phase packed in the 183-cm by 4-mm (inner diameter) glass column consisted of 17% ethylene glycol adipate polyester coated on 60/80 mesh, Chromosorb W.[9] The flash heater, column and detector were kept at 275°, 195° and 225°, respectively. Argon was used as the carrier gas at an inlet pressure of 14 psi. The amount of each fatty acid was quantitated by multiplying the peak height by its width at half-height. The results are expressed as the percentage fatty acid of the total by weight which is a function of the peak area produced by the fatty acid. For identification of tissue fatty acids, methyl ester derivative retention ratios (relative to palmitate) were compared with fatty acid methyl ester standards.[10] The linearity of the detector response was verified by determining the quantitative composition of a mixture of standards[11] of known composition.

RESULTS AND DISCUSSION

The total liver lipids increased from 37.2 to 49.8 mg (hydrogenated vegetable oil) or to 61.8 mg/g liver (safflower oil diet) during the duration of the experiments in which choline was omitted in the diet (fig. 1). As shown in the graph, the increase in total liver lipids is due to the increase in the neutral lipid fraction since during this time the phospholipid concentration decreased slightly. The increase in liver

[8] Model 10 Gas Chromatograph, Barber-Colman Company, Rockford, Illinois.
[9] Analytical Engineering Laboratories, Inc., Hamden, Connecticut.
[10] Applied Science Laboratories, Inc., State College, Pennsylvania.
[11] The Hormel Institute, Austin, Minnesota.

TABLE 2

Fatty acid composition of the dietary fats [1]

Fatty acid [2]	Laboratory chow [3]	Hydrogenated vegetable oil [4]	Safflower oil	Cod liver oil
12	tr [5]	tr	— [6]	tr
13	tr	—	—	—
14	2.3	tr	—	4.9
14:1 [7]	tr	—	—	tr
15	tr	—	—	tr
16:iso [7,8]	tr	—	—	tr
16	27.7	14.1	5.5	14.4
16:1	2.5	—	—	14.4
17	tr	—	—	tr
18	7.9	9.1	2.5	1.9
18:1	32.6	47.2	10.1	32.1
18:2	23.7	26.4	82.0	1.4
18:3	2.2	2.3	—	tr
20:1 [7]	—	—	—	13.7
20:5 [7]	—	—	—	4.9
20:un [7,9]	—	—	—	10.1

[1] Values are expressed as per cent of total by weight.
[2] Number of carbon atoms:number of double bonds.
[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[4] Crisco, Procter and Gamble Company, Cincinnati.
[5] Indicates trace quantity (less than 1%).
[6] Indicates not present.
[7] Tentative identification.
[8] Indicates iso, branched chain.
[9] Indicates unknown degree of unsaturation.

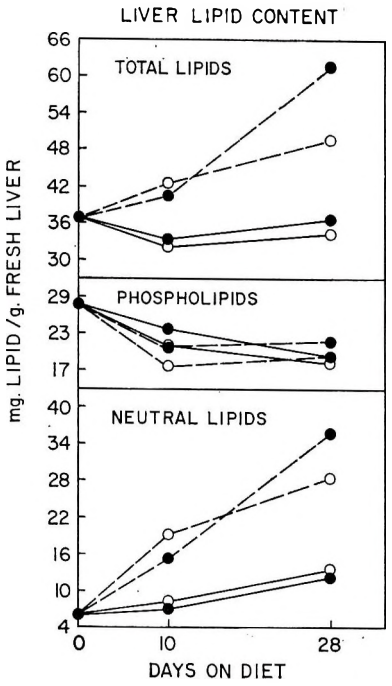

Fig. 1 Liver lipid composition of choline-deficient and choline-supplemented rats. ○, hydrogenated vegetable oil; ●, safflower oil; — — —, choline-deficient; ———, choline-supplemented.

neutral lipids occurred in choline-deficient animals when either the hydrogenated vegetable oil or safflower oil was used as the source of dietary fat. Choline supplementation of the low protein diet did not completely prevent the accumulation of neural lipids as this fraction increased slightly during the duration of the experiments.

The fatty acid composition of whole liver triglycerides, diglycerides and lecithin is shown in figures 2 through 4, with each point representing the data obtained from the livers pooled from 3 animals. Only the major fatty acids of these lipids are shown, although myristic, pentadecanoic, isopalmitic, hexadecatrienoic and heptadecanoic acids were present in small quantities (less than 2%). The changes in the fatty acid composition of the liver lipids observed during the 28 days were similar with respect to the type of fat used in the diet. No important differences were noted in the fatty acid composition between animals fed rations with or without the choline supplement, either when the hydrogenated vegetable oil or safflower oil was used as the dietary fat.

Generally, there was a steady increase of about 6 to 13% by weight in the palmitic acid content of all lipids under all dietary conditions in the 28-day duration of the experiments. This is most probably due to the high content of cabohydrate (sucrose and dextrin) in the diet since MacDonald (23) has shown that the palmitic acid level of liver lipids increases when animals are fed high sucrose diets. The palmitic acid content of the triglycerides tends to be slightly higher in the choline-deficient animals, whereas in the whole liver lecithin fraction the animals receiving the hydrogenated vegetable oil appeared to have a larger percentage of palmitate when compared with those consuming safflower oil.

The large increase in the palmitic acid levels is reflected by the increase of palmitoleic acid content of all fractions. The increase of palmitoleic acid, which amounts to 7% by weight in the triglycerides, may result from desaturase activity upon the increased level of palmitic acid. In all fractions in which the hydrogenated vegetable oil was used, the level of palmitoleic acid attained was higher than when safflower oil was utilized, but no large difference in the palmitoleic acid content of the lipids was detected between non-choline or choline-supplemented rations. Originally, the increase in the palmitoleic acid content of the lipid fractions was thought to be due to the high palmitoleic acid content (14.1%, see table 2) of the cod liver oil used in the diet. However, an experiment was carried out in which the cod liver oil was replaced by crystalline preparations of vitamins A and D.[12] The removal of cod liver oil from the diet did not prevent the increase of palmitoleic acid in the lipid fractions.[13]

The greatest differences in fatty acid patterns were observed with the changes of oleic and linoleic acid content of the liver lipids. When hydrogenated vegetable oil was used as the dietary fat the level of oleic acid increased markedly, especially in the diglyceride fraction with a 12% by

[12] See footnote 3.
[13] Unpublished data, E. A. Glende, Jr. and W. E. Cornatzer, 1965.

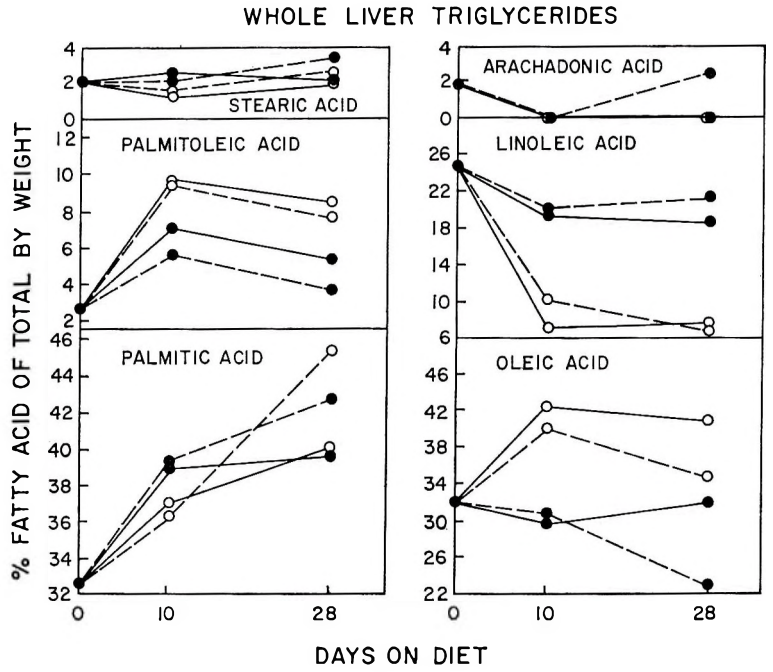

Fig. 2 Fatty acid composition of whole liver triglycerides of choline-deficient and choline-supplemented rats. ○, hydrogenated vegetable oil; ●, safflower oil; — — —, choline-deficient; ———, choline-supplemented.

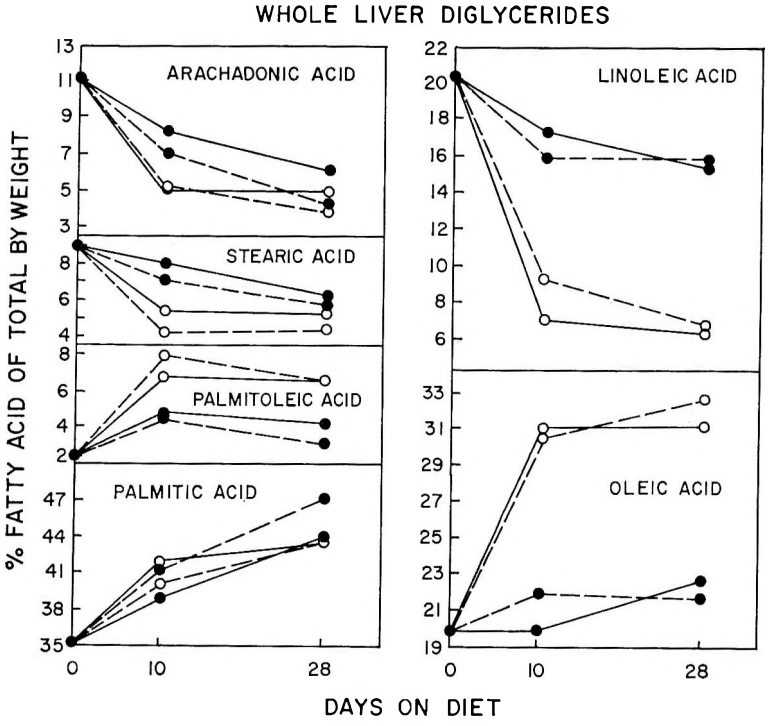

Fig. 3 Fatty acid composition of whole liver diglycerides of choline-deficient and choline-supplemented rats. ○, hydrogenated vegetable oil; ●, safflower oil; — — —, choline-defi-

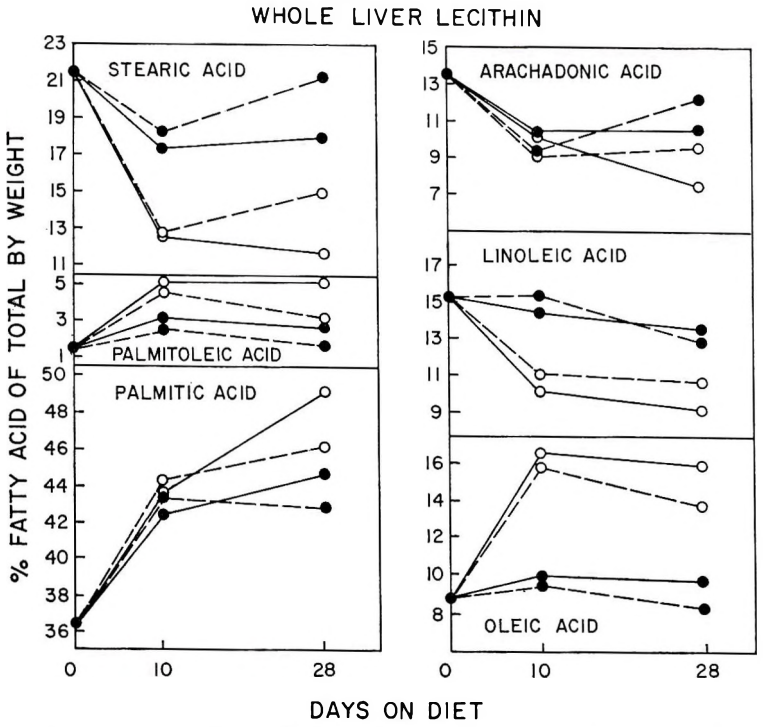

Fig. 4 Fatty acid composition of whole liver lecithin of choline-deficient and choline-supplemented rats. ○, hydrogenated vegetable oil; ●, safflower oil; — — —, choline-deficient; ———, choline-supplemented.

weight increase, and the amount of linoleic acid decreased markedly in the triglyceride and diglyceride fractions. However, when safflower oil was utilized the oleic acid level rose only slightly and only a small decrease in the linoleic acid content was noted. The slight decrease in linoleic acid in the safflower oil diet is also in accord with the observations of MacDonald (23) who showed that the liver linoleic acid content decreased as the dietary sucrose level was increased. As has been shown by a number of investigators, and observed again in these experiments, the changes in the oleic and linoleic acids of liver lipid is due to the variations of these fatty acids in the dietary fat (24–28). It is important to point out, nevertheless, that only with the exception of the greater decrease of oleic acid from triglycerides of choline-deficient rats as compared with choline-supplemented animals, there is essentially no difference with choline deprivation in the oleic and linoleic acid content of the whole liver lipids reported.

Since arachadonic acid is a product of the chain lengthening and further desaturation of linoleic acid (29, 30) it is not unexpected to observe that arachadonic acid decreased concurrently with the depression of the linoleic acid content. This decrease is especially marked in the diglycerides. Again, however, there is no difference between the arachadonic acid content of non-choline or choline-supplemented animals with the exception of the slight increase of arachadonic acid in the triglycerides of the animals fed a safflower oil, choline-deficient diet. The hydrogenated vegetable oil, which lowered linoleic acid levels more than safflower oil, also caused a slightly greater decrease in the arachadonic acid content of these 2 lipids.

The stearic acid content of liver triglycerides remained relatively constant at about 1 to 3% by weight, whereas in the diglyceride and lecithin fractions a decrease was observed. This decrease was greater in lecithin than in the diglycerides, especially with the hydrogenated vegetable

oil diet. In lecithin, after 28 days, the stearic acid level tended to increase to a small extent in both the hydrogenated vegetable oil and safflower oil, choline-deficient animals. With this exception no large differences in stearic acid content occurs in non-choline versus choline-supplemented rats.

Table 3 shows the fatty acid composition of whole liver and mitochondrial total phospholipids and mitochondrial lecithin. In general, the whole liver total phospholipid fatty acids exhibit changes similar to those observed in the neutral lipid and lecithin fractions. However, the stearic acid content of the whole liver total phospholipid fraction remained constant in the choline-deficient animals fed safflower oil, whereas with the other dietary regimens the stearic acid level decreased at 10 days and then increased again after the animals had been fed the diets for 28 days.

Changes in the fatty acid composition of mitochondrial phospholipids also resemble those of whole liver lipids. The differences noted in mitochondrial lecithin reflect that of the total mitochondrial phospholipids. As before, the changes produced in the fatty acid patterns of these lipids is most probably due to the carbohydrate and fat composition of the semi-purified diets rather than to the removal of choline.

Other workers have noted changes in the liver lipid fatty acids during dietary amino acid deficiencies. Lyman et al. (31) have recently shown that the fatty acid composition of liver triglycerides formed in a fatty liver during isoleucine deficiency differed slightly from that of isoleucine-supplemented animals in that palmitic acid in-

TABLE 3

Fatty acid composition of whole liver and liver mitochondrial phospholipids of normal and choline-deficient rats [1]

	Dietary fat	Dietary choline	Mitochondria						Whole liver		
			Total phospholipids			Lecithin			Total phospholipids		
Days fed diet			0 [2]	10	28	0 [2]	10	28	0 [2]	10	28
Palmitic acid	HVO [3]	+	22.7	32.7	35.2	40.2	47.0	48.6	25.8	36.0	38.8
	HVO	−		30.1	33.2		49.9	45.2		33.7	34.3
	SO [4]	+	22.7	30.1	30.8	40.2	43.5	46.5	25.8	36.6	35.5
	SO	−		29.7	30.8		41.5	44.0		33.2	34.8
Palmitoleic acid	HVO	+	1.1	6.4	4.7	1.4	6.0	4.3	1.0	4.3	4.0
	HVO	−		5.8	4.1		5.3	4.1		4.2	3.4
	SO	+	1.1	2.7	2.3	1.4	3.3	2.6	1.0	2.7	2.1
	SO	−		2.0	1.4		2.3	1.4		1.7	1.6
Stearic acid	HVO	+	18.8	13.5	15.0	20.9	13.0	13.3	23.1	17.4	18.1
	HVO	−		13.9	17.1		12.7	15.7		17.0	21.4
	SO	+	18.8	17.1	18.6	20.9	16.8	17.2	23.1	17.3	20.5
	SO	−		20.2	21.1		20.4	22.2		22.7	22.6
Oleic acid	HVO	+	8.7	14.5	14.0	8.0	16.5	15.4	7.8	12.9	12.4
	HVO	−		14.8	12.8		14.7	13.7		13.3	11.5
	SO	+	8.7	8.8	7.8	8.0	10.0	9.1	7.8	10.3	7.7
	SO	−		7.6	6.2		8.2	6.9		7.7	7.9
Linoleic acid	HVO	+	26.4	17.1	17.7	13.2	8.0	8.4	17.8	11.1	10.5
	HVO	−		19.3	18.7		8.0	9.5		12.4	12.4
	SO	+	26.4	24.0	23.0	13.2	13.6	12.4	17.8	16.4	15.7
	SO	−		23.3	22.1		13.9	10.6		14.8	13.8
Arachadonic acid	HVO	+	21.2	15.1	12.8	13.6	6.7	7.8	23.0	17.4	15.6
	HVO	−		15.5	13.7		5.6	10.1		18.6	16.6
	SO	+	21.2	16.7	17.1	13.6	10.8	10.8	23.0	15.3	18.1
	SO	−		16.7	17.5		11.7	13.1		19.4	18.7

[1] Values expressed as per cent of total by weight.
[2] Rats maintained with laboratory chow prior to the semi-purified diets.
[3] HVO, hydrogenated vegetable oil.
[4] SO, safflower oil.

creased and linoleic acid decreased in the deficient rats, whereas the liver phospholipid fatty acid composition remained unchanged. Viviani et al. (32) have reported that the weight percentage of linoleic acid is higher in the liver neutral lipid from a fatty liver of lysine- and threonine-deficient rats, whereas the amount of docosapentenoic and docosahexenoic acids in liver phospholipids is significantly lower in the deficient animals.

Kennedy (33) has demonstrated that diglycerides are intermediates in the biosynthesis of phospholipids and triglycerides. This observation is further substantiated by the similarity noted in the changes of fatty acid patterns of whole liver diglycerides (fig. 3) compared with whole liver lecithin (fig. 4) and triglycerides (fig. 2).

In this report we have attempted to study the fatty acid patterns of the major lipids involved in liver lipid metabolism with the intent to investigate whether the fatty acids of these compounds are involved in the abnormal lipid metabolism taking place during choline deficiency. Although the fatty acid content of liver lipids is influenced by the level of carbohydrate and the quality of fat in the diet, no significant alteration of liver lipid fatty acids is evident between animals fed rations with and without choline. It is unlikely that the small differences observed occasionally in the lipid fatty acid patterns account for the rather high content of neutral lipid that accumulates during dietary choline deficiency, and it may be concluded from these experiments that the fatty acid moiety of liver lipids does not appear to be responsible for the altered lipid metabolism of the liver during choline deficiency. Other factors such as diminished transport of fat from the liver due to lower serum lipoprotein levels (34) may cause the accumulation of liver fat in choline deficiency.

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Metabolism of Ethylenediaminetetraacetic Acid (EDTA) by Chickens [1]

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ABSTRACT Zn^{65} and EDTA-2-C^{14} were administered to 2 colostomized laying hens in a metabolic chamber which permitted the collection of CO_2 and excerta. EDTA-2-C^{14} was absorbed from the gastrointestinal tract and C^{14} from it was recovered in the respiratory CO_2, urinary uric acid and liver fat.

O'Dell and Savage (1) reported that zinc is required by chickens and that zinc in soybean protein is less available than that in casein and gelatin. Supplee et al. (2) observed zinc to be needed also by turkey poults and showed that the dietary requirement was reduced when the isolated soybean protein used in the diet was autoclaved. Kratzer et al. (3) reported better utilization of zinc not only by using autoclaved isolated soybean protein but also by the addition of EDTA to the diets. EDTA improved the utilization of zinc, manganese and copper by chickens from a diet containing isolated soybean protein (4, 5). Scott and Ziegler (5) postulated the existence of natural chelates in some food materials which also improved the utilization of zinc. Forbes (6) reported improved utilization of zinc by rats when the diet contained EDTA.

It has been suggested that phytic acid contained in the isolated soybean protein may be involved in making the zinc unavailable. Zinc or EDTA fed along with phytic acid largely counteracted the detrimental effect of phytic acid (7–8). Allred et al. (9) demonstrated the in vitro binding of zinc by isolated soybean protein and its release by EDTA. Isolated soybean protein, with two-thirds of its phytic acid removed, bound less zinc than untreated isolated soybean protein. The exact mechanism by which EDTA improves zinc utilization is not clear.

About 18 and 5% of an orally administered dose of Ca-EDTA-C^{14} was absorbed by rats and humans and less than 0.1% was respired as $C^{14}O_2$ by rats (10). Also, after an injection, 70% of the ionic zinc was retained by the rats in contrast with a retention of 12% of the dose if injected as Zn-Na_2EDTA (10). Stevens et al. (11) also observed an absorption of about 4 to 10% of EDTA-C^{14} by humans from an oral dose.

The purpose of the present work was to investigate the mechanism by which EDTA improves zinc utilization and to determine its absorption and metabolism by chickens.

METHODS AND MATERIALS

The apparatus used for the collection of CO_2 was essentially similar to the one described by MacKenzie et al. (12). A pressure cooker 35 cm in diameter and 35 cm in depth with a Plexiglass window was used as a metabolism cage.

The Zn^{65} activity was determined with a crystal scintillation detector after known amounts of samples had been dried on a polyethylene planchet. C^{14} activity was determined by wet combustion of the sample by the method of Van Slyke et al. (13) and counting the C^{14} as $BaCO_3$ under a windowless gas-flow counter. Self-absorption errors were compensated. Two experiments were conducted with colostomized Single Comb White Leghorn laying hens. The birds were fitted in a harness to which was attached a polyethylene bottle for urine collections and a rubber balloon for fecal collections. The birds in the 2 experiments were 18 months and 9

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months of age and weighed 1.5 and 2 kg, respectively. They were fed a zinc-deficient diet [3] containing isolated soybean protein (table 1) for three to six days and were transferred to the metabolism cage at least 26 hours before the radioactive materials were administered. $Zn^{65}Cl_2$ and EDTA-2-C^{14} solutions mixed together in gelatin capsules were administered orally. In each experiment approximately 5.1×10^6 counts/minute of Zn^{65} and 11.0×10^6 counts/minute of EDTA-2-C^{14} were administered per bird. The total amount of zinc in experiments 1 and 2 was approximately 0.0063 mmoles and of EDTA approximately 0.0074 mmoles. In the first experiment, blood samples were taken at various intervals and in the second experiment at the end of the experiment only. The red cells were washed several times with 0.9% saline solution and the washings added to the plasma fraction. Urine and feces were sampled at various times during the experiments. Urine was homogenized, made to volume with water and an aliquot was taken on a weighed plastic planchet for dry-matter determination and Zn^{65} counting. A second sample was taken for the C^{14} determination.

Feces were repeatedly extracted with 1.5% NH_4OH (14) to recover EDTA and the extract was made up to a known volume. The C^{14} activity was determined in the extract, and Zn^{65} activity was determined both in the extract and in the dry residue that was left after extraction. Respiratory CO_2 was collected for varying intervals of time in 1 N NaOH and aliquots were precipitated as $BaCO_3$ for counting.

Experiment 1 was continued for 48 hours and experiment 2, for 6 days.

Crude uric acid isolated from the urine by the method of Fisher (15) was washed with 50% alcohol and further purified by a procedure of St. John and Johnson (16). Purity of the sample was checked from its nitrogen content as determined by the micro-Kjeldahl method. The filtrate from the urine after the uric acid separation was concentrated by lyophilization or under reduced pressure. Non-labeled EDTA was added to this concentrate, and the solution was made alkaline with NaOH to assure the dissolution of EDTA and filtered. The filtrate was applied to a filter paper as a narrow band and developed by descending chromatography for 16 hours, using a solution containing equal parts of isopropyl alcohol and 0.2 N HCl. The paper was dried. The EDTA band was detected from its fluorescence under ultraviolet light as well as the blue color produced by spraying the paper with 5% $CuSO_4$ solution. The EDTA was found to be pure and uncontaminated with amino acids chromatographically. EDTA was eluted from the filter paper, dried and combusted for C^{14} determination.

TABLE 1

Zinc-deficient diet for laying hens

	%
Isolated soybean protein [1]	20.0
Soybean oil	6.0
Cellulose [2]	5.0
Cornstarch	59.63
Mineral mixture [3]	5.27
Limestone, ground	2.5
Vitamin A, D, E conc [4]	0.15
Choline chloride	0.1
Vitamin mixture [5]	0.15
$NaHCO_3$	1.0
DL-Methionine	0.2

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

[2] Solka Floc, Brown Company, Berlin, New Hampshire.

[3] Supplied in g/kg diet: $CaCO_3$, 3.0; $Ca_3(PO_4)_2$, 28.0; $K_2H(PO_4)_2$, 9.0; $MgSO_4 \cdot 7H_2O$, 2.5; $FeSO_4 \cdot 7H_2O$, 0.715; KI, 0.04; $CuSO_4 \cdot 5H_2O$, 0.02; H_3BO_3, 0.009; $Co(Ac)_2 \cdot 4H_2O$, 0.00063; $MnSO_4$, 0.65; and NaCl, 8.8.

[4] Supplied per kg diet: vitamin A, 10,000 IU; vitamin D_3, 600 ICU; and α-tocopheryl acetate, 5 IU.

[5] Supplied in mg/kg diet: thiamine·HCl, 25; riboflavin, 16; Ca pantothenate, 20; vitamin B_{12}, 0.02; pyridoxine·HCl, 6; biotin, 0.6; folic acid, 4; inositol, 100; p-aminobenzoic acid, 2; menadione, 5; ascorbic acid, 250; and niacin, 150.

RESULTS

The absorption of EDTA was rapid with a rather high activity of C^{14} in the blood even 45 minutes after the administration of the radioactive dose to chickens (table 2). The ratio of C^{14} to Zn^{65} was about double this value in the orally administered dose during the first 75 minutes but subsequently reached one-half this value. It appears that C^{14} from EDTA is absorbed at a faster rate than Zn^{65} for an initial period. The subsequent lowering of this ratio probably indicates the accumulation of zinc and the metabolism and excretion

[3] We are grateful to Merck and Company, Rahway, New Jersey, American Cyanamid Company, Pearl River, New York, and the Dow Chemical Company, Midland, Michigan, for various dietary supplements.

TABLE 2

C^{14} and Zn^{65} activities in the whole blood after feeding EDTA-2-C^{14} and Zn^{65} to a laying hen (exp. 1)

Time after administration of labels	C^{14}	Zn^{65}	$\frac{C^{14}}{Zn^{65}}$
hours:min	*count/min/ml*		
0:45	762	152	5.0
1:15	1712	288	6.0
4:45	270	534	0.5
8:00	365	561	0.65
12:00	520	406	1.3
24:00	443	337	1.3
36:00	340	328	1.0
48:00	264	277	1.0
Original	11×10^6	5.1×10^6	2.2

of EDTA with a lowering of the C^{14} in the blood. The C^{14} activity was observed mainly in the plasma, confirming the observation of Foreman (10), and Foreman and Trujillo (14), using rats and human beings in which they observed that EDTA did not penetrate red blood cells. The Zn^{65} was noted both in the red cells and plasma.

The pattern of C^{14} excretion in the respiratory CO_2 is shown in figure 1. In experiment 1 the specific activity of CO_2 was still increasing at 42 hours which prompted the extension of the experimental period to 144 hours in the second experiment. In both of these experiments, the C^{14} activity reached a peak at 7 hours. The other peaks probably reflect the utilization of certain metabolic end products of EDTA. The C^{14} was present in the respiratory CO_2 even at 110 hours after the administration of EDTA-2-C^{14} in experiment 2. The metabolism of EDTA-2-C^{14} in these 2 experiments appeared markedly different than the metabolism of acetate-2-C^{14} by a chicken [4] in which a single peak of C^{14} in the respiratory CO_2 was reached much earlier.

The C^{14} activity in the urine of the chicken reached a maximal value at about 11 hours in experiment 1 (fig. 2). It was difficult, however, to locate the peak accurately in the second experiment since no urine was passed between 2.5 and 12 hours and between 12 and 30 hours after

[4] Unpublished data.

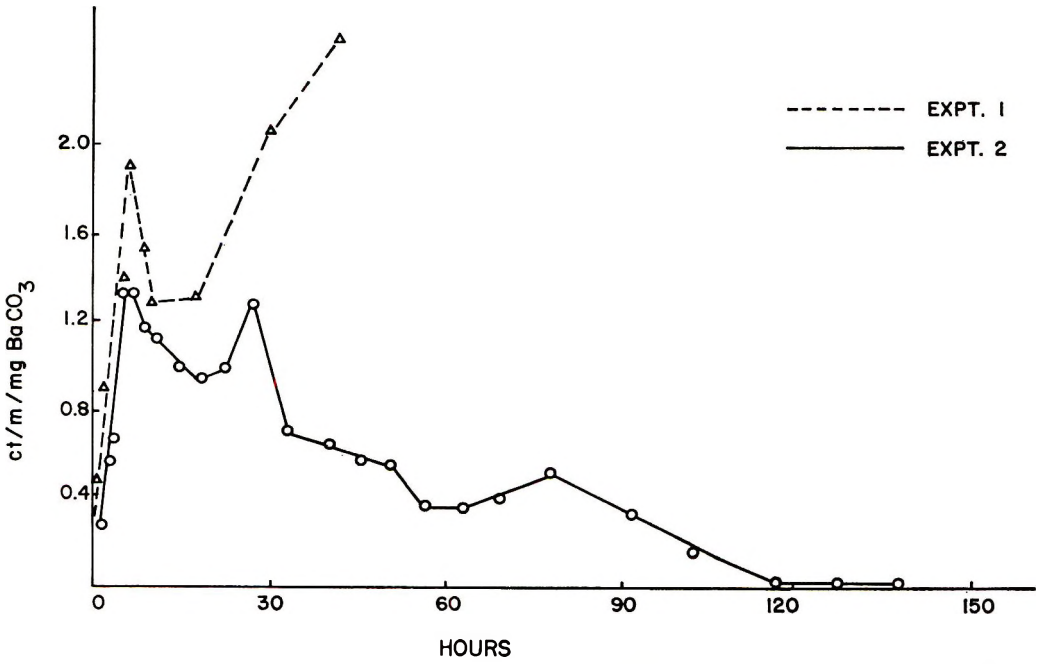

Fig. 1 Respiratory $C^{14}O_2$ after the oral administration of EDTA-2-C^{14} and $Zn^{65}Cl_2$ to laying hens.

the dose administration. A low level of C^{14} activity was still being excreted 144 hours after the dose was administered in experiment 2. Zn^{65} excretion in the urine paralleled the excretion of C^{14} in experiment 2, although at a somewhat lower level.

Some C^{14} activity was associated with the uric acid samples isolated from urine, yet 73 and 67% of the C^{14} activity was observed in the EDTA in the 2 experiments, respectively. Although bile was not collected quantitatively, samples of it in both experiments 1 and 2 contained both C^{14} and Zn^{65} activity.

Calculations for the recovery of administered carbon (table 3) indicate that 4.2 and 5.4% of the carbon was accounted

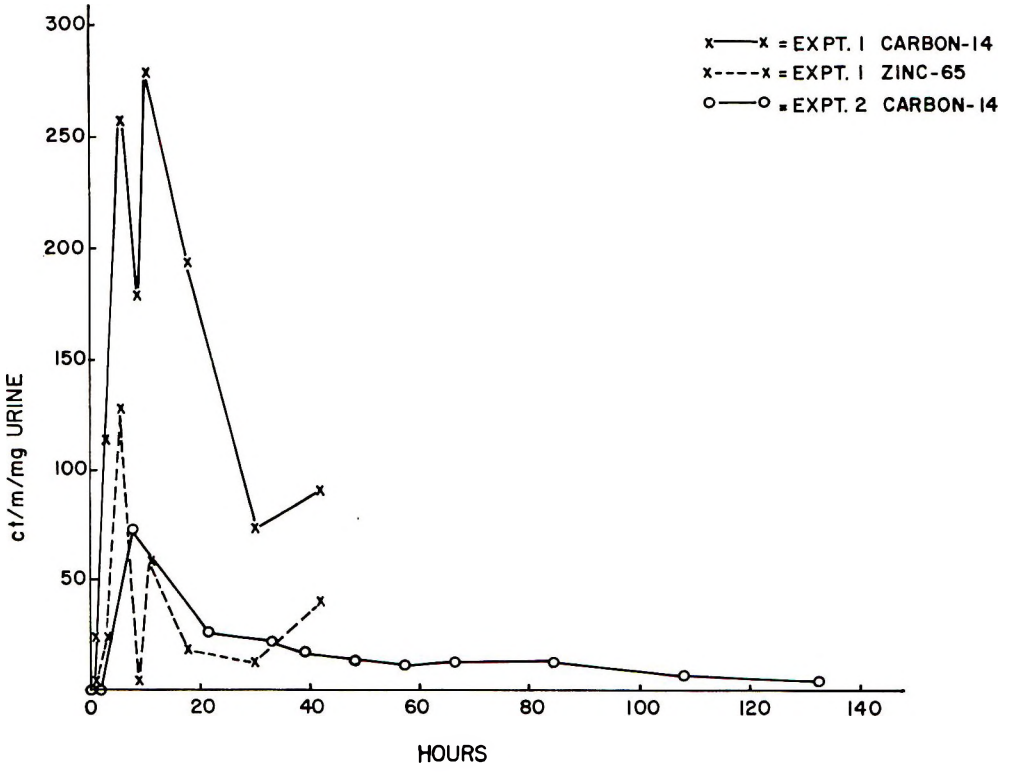

Fig. 2 C^{14} and Zn^{65} in the urine of laying hens after the oral administration of EDTA-2-C^{14} and $Zn^{65}Cl_2$.

TABLE 3

C^{14} and Zn^{65} recovery as percentage of dose after administration of EDTA-2-C^{14} and $Zn^{65}Cl_2$ to laying hens

	C^{14}		Zn^{65}	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Duration of trial, hours	48	144	48	144
1 CO_2	5.4	4.2		
2 Urine	10.9	8.6	4.7	0.19
3 Feces	29.2 (to 36 hr)	51.5	45	90.3
4 Gastrointestinal tract washing	25.6	1.4	20.1	0.9
5 Feces + gastrointestinal tract washing (3+4)	54.8	52.9	65.1	91.2

for by CO_2 in the 2 experiments, whereas 8.6 and 10.9% was observed in the urine. The amount in the feces differed somewhat in the 2 experiments due to the differences in duration of the trials. However, when the amounts in the feces and in the gastrointestinal tract washings were combined, the totals were comparable, slightly greater than 50%. About 45 and 47% of the C^{14} activity of EDTA remained in the body of the chickens. Recovery of Zn^{65} in the urine was 4.7 and 0.2% of the administered dose, whereas the amount in the feces and in the gastrointestinal tract washings was 65 and 91%. It is probable that the differences were due to variability in the rates of absorption of zinc by the 2 experimental chickens. It appears that the administered Zn^{65} was absorbed and eliminated at a much slower rate than the C^{14} from EDTA.

DISCUSSION

A hypothesis may be proposed for the role of EDTA in improving the availability of zinc. EDTA, a chelating agent, can remove zinc from its complexes with dietary proteins and form an EDTA-Zn complex. The size of the EDTA-Zn complex molecule may be much smaller than the protein-Zn complex and permit its absorption through the gastrointestinal tract. In blood and tissues EDTA may release zinc to proteins and enzymes which have greater affinities for zinc than EDTA. This mechanism could not be accepted on the basis of Foreman's work with rats and humans in which EDTA as its calcium complex was poorly absorbed and metabolized from an oral dose.

By the use of oral doses of equal molecular quantities of Zn^{65} Cl_2 and EDTA-2-C^{14} with colostomized laying hens, it was thought that a comparison of the ratio of C^{14} and Zn^{65} in the blood with that of the given dose would indicate whether Zn^{65} was absorbed as the EDTA-Zn complex. From the differences in the C^{14}-to-Zn^{65} ratios in the blood and oral dose, it appeared that C^{14} from EDTA and Zn^{65} from equal molar quantities of an oral dose were absorbed at somewhat different rates, possibly influenced by the presence of other ions in the complex biological system. It was difficult to establish from these experiments whether zinc in the presence of EDTA was actually absorbed in the gastrointestinal tract as the EDTA-Zn complex. It might be concluded from the experiments that EDTA was absorbed and metabolized by chickens, in contrast with studies with rats,[5] and the C^{14} was recovered in the respiratory CO_2, liver fat and urinary uric acid.

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Mineral Utilization in the Rat

V. EFFECTS OF DIETARY THYROXINE ON MINERAL BALANCE AND TISSUE MINERAL COMPOSITION WITH SPECIAL REFERENCE TO MAGNESIUM NUTRITURE

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ABSTRACT Two experiments were conducted, using a total of 220 young male albino rats, to study the effects of thyroxine on the response to varying intakes of Mg. Weight gain, tissue mineral composition, mineral balance, serum and kidney glycoprotein were criteria of response. Thyroxine reduced the weight gain but did not influence the Mg required for optimal weight gain at a given thyroxine level, nor did it affect the Mg required for normal levels of Mg in blood serum or in bone ash; however, thyroxine did prevent the kidney calcification normally occurring in Mg-deficient animals but did not prevent calcification of heart muscle. Blood serum glycoprotein was reduced by thyroxine although kidney glycoprotein was not affected. Balances of Ca, P and Mg were reduced by thyroxine, mainly as a result of reduced apparent absorption of these minerals.

It has been reported previously (1) that markedly increased dietary levels of Mg are required to support maximal weight gains and to prevent hyperemia of the extremities and decrease in blood serum Mg levels in young rats fed diets containing thyroxine. The conclusion that thyroxine increases the Mg requirement is supported by the observation that, both in vitro and in vivo, thyroxine uncouples oxidative phosphorylation in intact mitochondria and that Mg restores this function (1–3). Nevertheless, not all investigators have successfully demonstrated an effect of Mg deficiency on mitochondrial oxidative phosphorylation (4). It has also been demonstrated that, although appearing to increase most of the sequelae of Mg deficiency, thyroxine markedly inhibits the characteristic deposition of Ca in the kidney tubules (3, 5). The effects of thyroxine administration on mineral balance have been reported to include decreased balance of Ca due to increased fecal output (6) and to increased urinary loss (7, 8). Aub (8) also reports the increased urinary Ca to accompany increased urinary P, but Rawson (9) reported urinary Ca not to be changed, whereas urinary P was increased markedly by thyroxine administration.

The following experiments were undertaken with the aim of studying further the effects of Mg and of thyroxine on mineral balance and tissue mineral composition, together with observations on the effect of thyroxine on the Mg requirement.

METHODS

Two experiments were conducted in this study, using a basal diet common to both and having the composition shown in table 1. Additions to the basal diet were made at the expense of glucose. The basal diets contained, by analysis, 0.50% of Ca and of P. Weanling male albino rats of Sprague-Dawley strain were used in both experiments. The rats were assigned at random to treatment and to cage location.

In experiment 1, additions of $MgCO_3$ and of L-thyroxine provided 5 levels of Mg (80, 170, 280, 490 and 970 ppm), each with and without 5 ppm thyroxine. The 80-ppm basal level of Mg was chosen as one which would be slightly inadequate for production of maximal weight gain. Ten rats were individually fed each diet for a period of 4 weeks. During each of weeks 3 and 4, 20 rats, two from each diet, were placed in metabolism cages for collection of urine

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TABLE 1
Composition of the basal diet used in both experiments

	%
Casein [1]	15.0
DL-Methionine	0.5
Corn oil	8.0
Cellulose	3.0
Salts [2]	2.66
Vitamin mix [3]	5.0
Vitamin A and D concentrate [4]	0.5
Glucose	65.34

[1] "Vitamin-Free" Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Contained: (in %) NaCl, 14.02; K_2CO_3, 18.94; $FeSO_4 \cdot 7H_2O$, 1.50; $MnSO_4 \cdot H_2O$, 0.45; $CuSO_4 \cdot 5H_2O$, 0.23; KI, 0.01; $ZnCO_3$, 0.07; $MgCO_3$, 0.75; and $CaHPO_4$, 64.02.
[3] Forbes, R. M., J. Nutrition, *83*: 225, 1964.
[4] Cod liver oil containing 0.6 mg vitamin A and 250 USP units vitamin D/g.

and feces. Hence, 4 rats per dietary treatment were used in this phase of the experiment. During the collection period all rats consumed 9 to 10 g of feed daily. When collections were not being made, the rats were fed ad libitum, and during the entire experiment deionized water was available. At the end of the experiment the rats were decapitated. Blood samples were taken and tibias and kidneys saved for analysis.

In experiment 2, additions of $MgCO_3$ and of L-thyroxine provided 5 levels of Mg (95, 185, 370, 690, and 1430 ppm) at each of 4 levels of thyroxine (zero, 5, 10, 20 ppm). Three groups of 40 rats each were placed on experiment in 3 consecutive 4-week periods, with 2 rats fed each diet in each period. The rats were control-fed for 3 weeks with excreta collections during week 3 when the rats were consuming 10 to 11 g of feed daily. They were then fed ad libitum for the 4th week. At the termination of the experiment blood samples, tibias, kidneys and hearts were obtained after decapitation.

Chemical analyses for Ca and Mg in experiment 1 were first made by chelometric titration and later most of these were checked by atomic absorption. In experiment 2, analyses for Ca, Mg, and K were made by atomic absorption. One kidney from each rat in experiment 1 was preserved in buffered 10% formalin, and after sectioning was stained with Calcon in accord with the principles used by Kaufman and Adams (10). The other soft tissues were preserved by freezing until they could be prepared for chemical analysis. In experiment 2 kidney and blood serum glycoprotein analyses were conducted by the method of Winzler (11); the results are expressed as milligrams of protein-bound hexose. Mineral analyses were conducted on soft tissues after determination of their dry-matter content and subsequent wet-ashing with nitric acid followed by hydrogen peroxide. Bone mineral analyses were made after dry-ashing the ethanol-petroleum ether extracted bone.

The data were treated statistically by standard analysis of variance procedures.

RESULTS

Experiment 1

The general appearance of the animals was not affected by the thyroxine dosage used. The thyroxine-treated animals were more active, but they showed no more severe symptoms of Mg deficiency than did the controls. In both groups erythema of the ears first appeared in 8 to 10 days in the rats fed at the basal level of Mg. This was followed by development of facial edema and scattered areas of skin lesions. Of the 20 rats fed at the 170-ppm Mg level, two showed a transient erythema of the ears at day 13; these were not animals treated with thyroxine.

The quantitative data of experiment 1 are shown in table 2. Urinary excretion data not shown in the tables may be calculated as the difference between absorption and balance.

Weight gain. All levels of Mg supplementation resulted in weight gains greater than did the basal level, but statistically significant differences are not present between the gains of the Mg-supplemented groups. Thyroxine administration significantly lowered the weight gain over the experiment as a whole but within Mg levels the difference was significant only in animals receiving the lowest level of Mg. Although not shown in the table, the feed intake of the rats was not influenced by thyroxine intake but decreased in those animals fed at the basal level of Mg. Statistical adjustment of gains to equal food intake removed the Mg effect on gain and resulted in average 28-day gains of 126 and 106 g for non-treated and thyroxine-treated rats, respectively.

TABLE 2

Effect of magnesium and thyroxine on weight gain, mineral balance and tissue mineral concentration [1] *(exp. 1)*

	Thyroxine, ppm	Magnesium, ppm					*P* values for treatment		
		80	170	280	490	970	Mg	T	Mg × T
Weight gain, g/28 days	0	86	127	132	145	129			
	5	65	108	114	125	129	0.01	0.01	NS [2]
Serum Mg, mg/100 ml	0	0.86	0.96	1.55	1.81	2.01	0.01	0.01	0.05
	5	0.83	1.29	1.91	1.97	1.95			
Bone ash Mg, %	0	0.27	0.45	0.72	0.78	0.84	0.01	0.01	NS
	5	0.34	0.48	0.75	0.81	0.90			
Kidney Ca % of dry matter	0	0.637	0.817	0.059	0.040	0.054	0.01	0.01	0.01
	5	0.164	0.060	0.044	0.054	0.058			
Ca absorbed, %	0	78	75	84	84	69			
	5	61	71	70	80	70	0.01	0.01	NS
Ca balance, %	0	77	73	81	81	65			
	5	60	67	67	77	68	0.02	0.01	NS
Mg absorbed, %	0	84	84	82	84	64			
	5	69	81	81	86	81	0.01	NS	0.01
Mg balance, %	0	74	66	47	25	19	0.01	0.02	0.01
	5	45	47	44	32	23			
P absorbed, %	0	90	85	89	90	79			
	5	79	82	82	87	81	0.02	0.01	NS
P balance, %	0	47	51	53	59	55	0.01	0.01	NS
	5	33	43	46	54	46			

[1] Ten rats/observation for gain and tissue analysis; four rats/observation for mineral balance.
[2] NS indicates nonsignificant.

Serum Mg. Dietary supplements of Mg increased the serum Mg level. Thyroxine-treated rats had increased serum Mg in comparison with non-thyroxine treated animals at the 170 and 280 ppm Mg levels, but not at lower or higher ones.

Bone ash Mg. The concentration of bone ash Mg was increased both by dietary Mg and thyroxine supplements, there being no evidence of a differential response to Mg in the presence or absence of thyroxine.

Kidney Ca. The usual kidney calcification accompanying low Mg diets was observed in the animals not treated with thyroxine. This was largely eliminated by thyroxine treatment. Histological examination of the kidneys corroborated the chemical analysis. Sharply delineated areas of calcification were observed in the corticomedullary zone. An arbitrary scoring system showed a good correlation between chemical and histological methods of rating degree of calcification. Expressed as a percentage of body weight, the dry kidney weight was increased by thyroxine treatment and decreased by Mg supplementation.

Ca absorption and balance. Only 1 to 4% of the Ca intake was excreted in the urine, and this variation was not associated with treatment. Thus the treatment effects on Ca absorption and balance are similar. The data show irregular differences in Ca absorption and balance between Mg levels and that thyroxine lowered Ca absorption and balance. The thyroxine effect was most evident in animals fed the 80- and 280-ppm Mg diets and was slight at other levels.

Mg absorption and balance. Except for the low absorption by rats receiving the highest level of Mg without thyroxine and the lowest level of Mg with thyroxine, treatment did not markedly affect Mg absorption. Urinary Mg varied from 10 to

59% of the intake and increased as dietary Mg increased. Hence the percentage Mg balance decreased with each supplement of Mg. The presence of thyroxine stimulated urinary Mg excretion at the 2 lower Mg levels, hence the Mg balance was adversely affected by thyroxine in these instances.

P *absorption and balance.* The pattern of P absorption followed that of Ca. Mg deficiency had no important effect on P absorption but did decrease P balance by increasing the urinary P. The presence of thyroxine increased urinary P and hence decreased P balance at all levels of Mg.

Experiment 2

The second experiment was undertaken as the result of certain inconsistencies in the mineral balance data of experiment 1 and to investigate the effects of several levels of thyroxine. As in the first experiment the time of appearance and the severity of Mg deficiency symptoms were not influenced by the presence of thyroxine and were prevented by Mg supplements. The quantitative data of experiment 2 are shown in tables 3 and 4.

Weight gain. The first level of Mg supplementation resulted in a marked increase in weight gain which was not influenced further by higher Mg levels. Thyroxine decreased weight gains, most of the effect being noticeable at the first thyroxine level, although each succeeding level progressively reduced weight gain. Feed intake, as in the first experiment, was not significantly affected by thyroxine but was reduced in groups fed at the lowest Mg level.

Serum Mg. The basal level of Mg resulted in reduced serum Mg levels. The thyroxine had no significant effect on serum Mg; at all thyroxine levels, 370 ppm Mg produced "normal" levels of serum Mg.

Kidney Ca. Prevention by thyroxine of the kidney calcification encountered in Mg deficiency is evident. At the lowest level

TABLE 3

Effect of thyroxine and magnesium on weight gains and tissue composition [1] *(exp. 2)*

	Thyroxine, ppm	Magnesium, ppm					P values for treatment		
		95	185	370	690	1430	Mg	T	Mg × T
28-Day wt gain, g	0	117	147	148	149	143			
	5	82	110	98	101	106			
	10	69	87	91	100	97	0.01	0.01	NS [2]
	20	67	83	81	75	83			
Serum Mg, mg/100 ml	0	0.90	1.77	2.16	2.33	2.39			
	5	1.03	1.82	2.15	2.36	2.72			
	10	1.12	1.76	2.27	2.33	2.48	0.01	NS	NS
	20	1.31	1.90	2.23	2.31	2.63			
Kidney Ca, % of dry matter	0	1.417	0.505	0.051	0.057	0.043			
	5	0.085	0.039	0.041	0.040	0.051	0.01	0.01	0.01
	10	0.064	0.045	0.039	0.040	0.047			
	20	0.047	0.042	0.037	0.039	0.044			
Bone ash Mg, %	0	0.28	0.59	0.85	0.83	0.92			
	5	0.41	0.67	0.84	0.89	0.96			
	10	0.43	0.67	0.83	0.89	1.00	0.01	0.01	0.01
	20	0.51	0.73	0.82	0.86	0.96			
Serum glycoprotein, mg hexose/100 ml	0	135	149	134	150	139			
	5	102	128	105	119	116	NS	0.01	NS
	10	103	103	104	113	96			
	20	92	98	84	107	98			
Heart Ca, % of dry matter	0	0.046	0.031	0.109	0.038	0.026			
	5	0.066	0.030	0.031	0.026	0.026	0.01	NS	0.01
	10	0.055	0.028	0.061	0.022	0.028			
	20	0.219	0.028	0.033	0.028	0.025			

[1] Six rats/observation.
[2] NS indicates nonsignificant.

TABLE 4

Effect of thyroxine and magnesium on mineral balance [1] *(exp. 2)*

	Thyroxine, ppm	Magnesium, ppm					*P* values for treatment		
		95	185	370	690	1430	Mg	T	Mg × T
Ca absorbed, %	0	81	79	72	78	74			
	5	60	63	65	65	59			
	10	60	58	59	64	55	0.01	0.01	NS [2]
	20	60	58	51	56	53			
Ca balance, %	0	79	77	68	76	71			
	5	57	61	62	61	55	0.01	0.01	NS
	10	58	56	54	62	54			
	20	56	56	49	53	51			
Mg absorbed, %	0	84	86	84	83	69			
	5	74	73	78	76	70	0.01	0.01	NS
	10	69	70	75	73	66			
	20	63	67	70	67	65			
Mg balance, %	0	75	65	34	21	13			
	5	63	50	28	5	10	0.01	0.01	0.05
	10	49	38	20	3	9			
	20	49	43	18	8	8			
P absorbed, %	0	89	88	82	86	79			
	5	79	77	75	75	69			
	10	76	73	73	75	63	0.01	0.01	NS
	20	74	70	65	67	64			
P balance, %	0	53	52	51	50	47			
	5	40	36	38	38	38			
	10	37	34	35	36	31	NS	0.01	NS
	20	29	33	25	30	30			

[1] Six rats/observation.
[2] NS indicates nonsignificant.

of dietary Mg, each thyroxine supplement decreased kidney calcification, although most of the effect was obtained with the 5-ppm thyroxine level. Kidney K averaged 1.26% of the dry matter and was uninfluenced by treatment.

Bone ash Mg. As with blood serum Mg, 370 ppm of dietary Mg produced "normal" bone ash Mg values irrespective of thyroxine levels. In the case of the bone analyses the tendency for thyroxine to increase Mg deposition at the lowest Mg level was statistically significant. This same tendency was present, but not statistically reliable, in the blood serum Mg values.

Blood serum glycoprotein. Mg supplementation did not affect the blood serum glycoprotein levels but they were lowered in animals receiving thyroxine supplementation. Kidney glycoprotein averaged 213 mg/100 g of fresh kidney and was uninfluenced by treatment.

Heart Ca. At all levels of thyroxine, heart Ca was increased in those animals fed at the basal level of Mg. The high average figure for the hearts of rats fed the highest thyroxine and lowest Mg diets is largely the result of 2 outstandingly high values of 0.64 and 0.33%. Heart K averaged 1.31% of the dry matter and was not influenced by treatment.

Ca absorption and balance. The major effect on Ca absorption and balance was a decrease accompanying the inclusion of thyroxine in the diet. The statistical difference observed between Mg levels is a result of lower values for Ca absorption and balance of those animals receiving the 370- and 1430-ppm Mg diets.

Mg absorption and balance. The major effect on Mg absorption was the decrease accompanying inclusion of thyroxine in the diet, although the highest level of dietary Mg was less completely absorbed than the lower levels. Mg balance was likewise

reduced by thyroxine, mainly an effect of inefficient absorption. The sharp reduction in Mg balance with increased dietary Mg was largely a reflection of increased urinary excretion of Mg.

P absorption and balance. The P absorption was reduced consistently by thyroxine and also by the highest level of Mg. The P balance was also reduced by thyroxine but, as a result of reduced urinary P of those animals receiving the highest level of Mg, the P balance was not significantly affected by Mg level.

DISCUSSION

The results of the 2 experiments reported here are in agreement in most respects. The evidence concerning the effects of thyroxine on the Mg requirement indicates that, according to the criteria of weight gain and concentration of Mg in blood serum and bone ash, the requirement is not changed appreciably. Although no attempt was made to accurately access the requirement in these studies, the data are consistent with our previous estimates (12). In the current investigation the requirement for weight gain did not exceed 170 ppm and that for normal levels of Mg in blood serum and bone ash approximated 400 ppm Mg. These data are at variance with those reported by Vitale et al. (1) which indicated that 20 ppm thyroxine increased the requirement for Mg for weight gain from less than 200 ppm to more than 400 ppm, and that this same level of thyroxine decreased serum Mg from 1.87 to 0.50 mg/100 ml in rats receiving 200 ppm Mg in the diet. In another report from the same laboratory (13) it appeared that thyroxine did not increase the Mg requirement for weight gain or for maintenance of serum Mg levels. Also, Corradino and Parker (14) did not observe an effect of dietary thiouracil on Mg requirement for weight gain of young rats, although rats treated with thiouracil that gained slowly did not show the typical visible symptoms of Mg deficiency.

Our data relative to the protective effect of dietary thyroxine supplements on the kidney calcification typically accompanying Mg deficiency are in complete agreement with previously published reports (3, 5), but the mechanism whereby this effect is brought about remains obscure. Histological studies of Hess et al. (15) showed the initial reaction of the kidney to Mg deprivation to be swelling and increased enzymic activity of mitochondria in the proximal convoluted tubules. This was followed by epithelial necrosis accompanied by appearance of fine Ca deposits. It seems probable that the thyroxine effect is mediated through prevention of the early changes such as integrity of the mitochondrial membrane rather than through a direct effect on calcification.

The glycoprotein analyses were made in an attempt to determine whether this class of material was increased demonstrably in the calcified kidneys of Mg-deficient animals. An increase in histologically detectable amounts of glycoprotein preceded the calcification of kidney induced by parathyroid hormone; at the same time blood serum levels of glycoprotein were increased from a normal of 165 to 233 mg/100 ml.[1] Contrary to the effect of parathyroid hormone, thyroxine in this experiment had no effect on kidney glycoprotein as determined chemically but did markedly decrease levels of serum glycoprotein from 141 mg/100 ml for the control animals to 96 mg/100 ml for those receiving 20 ppm thyroxine. The significance of this previously unreported effect of thyroxine is unknown, but the observation may indicate that a proper balance between thyroxine and parathormone may be a factor in the synthesis of serum glycoproteins in the liver or in their transport or catabolism.

The bone ash Mg showed the same trends toward increase in response to thyroxine in both experiments although the effects reached significance only in experiment 2 in which the thyroxine-treated animals fed at the lower levels of Mg had increased bone ash Mg. This effect is probably a reflection of the larger intakes of total feed and hence of Mg per unit body weight on the part of the thyroxine-fed animals and is in agreement with the same tendency noted as a result of cold stimulus to feed intake (12). The lack

[1] Grob, H. S. 1962 A study of the actions of estradiol and parathyroid extract on some phases of calcium metabolism in the albino rat. Ph.D. Thesis, New York University.

of effect of Mg intake on heart and kidney K is at variance with results of Whang and Welt (16) and Manitius and Epstein (17), probably because the Mg deficiency produced in this study was less severe.

Since the Ca and P intakes did not vary appreciably between treatments during the week of collection, the percentage balance of these nutrients is proportional to the absolute amounts retained. Supplements of Mg did not have a consistent effect on Ca balance, but the thyroxine supplements uniformly lowered it. In general, P absorption and balance followed the same pattern as that for Ca. Urinary excretion of Ca was not affected by treatment but that of P was increased by thyroxine and decreased by the highest level of Mg. Alcock and MacIntyre (18) reported that Ca absorption was greater in Mg-deficient than in normal rats and that the deficient animals excreted less Ca in the urine. Their basal diet (6 ppm Mg) was more deficient than that used in this study (95 ppm). This same fact may explain the lack of hyperphosphaturia in the present study as compared with the marked increase in urine P of Mg-deficient rats reported by Smith et al. (19). The decreased balance of Ca and P accompanying thyroxine administration is generally compatible with previously published results (6, 9) although other data (7–9) obtained with human subjects, indicate an increased urinary excretion of Ca and P.

Mg absorption was depressed at all levels of supplementary thyroxine but by Mg supplements only at the highest Mg concentration, conditions under which Ca and P absorption also decreased. Thus the apparent absorption of these 3 elements was affected similarly by the treatments used. Urinary Mg was markedly increased by each Mg supplement; hence the balance of Mg was sharply reduced. Thyroxine supplements did not affect the urinary Mg, in agreement with their effect on urinary Ca but in contrast with their effect of increasingly urinary P.

It appears likely that the effects of thyroxine on Ca, Mg and P balances are in part indirect ones, mediated through the general metabolic stimulus of thyroxine and the relative deficiency in energy intake in the thyroxine-treated animals. The energy deficiency is apparent from the data relative to weight gain, as the total feed intake was not affected by thyroxine but weight gains were reduced. In corroboration of the Ca and P balances, thyroxine supplements decreased the weight of tibia ash, which averaged 213, 177, 165 and 150 mg, respectively, as the thyroxine dosage increased. Evidence for effect of thyroxine in reducing net absorption of Ca by everted gut sacks has been provided by Finkelstein and Schachter (20).

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Pantothenic Acid Deficiency in Pregnant and Non-pregnant Guinea Pigs, with Special Reference to Effects on the Fetus [1,2]

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ABSTRACT To study the effects of pantothenic acid deficiency during pregnancy in the guinea pig, it was necessary first to establish the appropriate duration of the deficiency period, by subjecting adult animals to the deficient diet. These experiments showed that the adult guinea pig is sensitive to a deficiency of pantothenic acid. Thirteen non-pregnant adults died within 10 to 41 days after institution of the deficient diet, and 3 pregnant animals survived only 9 to 16 days. Liver pantothenic acid and coenzyme A levels were markedly reduced, and liver fat concentration was greatly increased. Pair-feeding experiments showed that this increase in liver fat was at least partially due to decreased food intake. A transitory dietary deficiency of pantothenic acid during the ninth or tenth weeks of gestation (total period 70 days) resutled in loss of fetuses through abortion, or death of the mother. Deficiency during the tenth week appeared to produce a significant increase in the liver fat of the newborn. Concomitantly, liver pantothenic acid level was lower than normal, both at birth and at 7 days of age. Young whose mothers had received the deficient diet during the ninth, the seventh or the sixth weeks showed no significant changes in liver fat at birth, but liver pantothenic acid concentration was lower than normal. No gross morphological abnormalities were noted. Liver fat concentration in normal newborn guinea pigs was found to be more than 10 times the normal adult level. At 62 days of gestation, it was even higher than at birth, but it decreased after parturition to one-third of the newborn level by 2 days of age. In contrast, liver cholesterol showed only slight changes in the neonatal period. The experiments suggest that the greatest need for pantothenic acid during fetal development of the guinea pig is in the period shortly before birth.

Pantothenic acid deficiency during gestation in the rat is known to result in offspring with a variety of congenital malformations (1–3). However, the biochemical changes underlying these anomalies are not known. The present paper reports the results of a study of pantothenic acid deficiency in the pregnant guinea pig, especially in terms of biochemical changes in the young.

Pantothenic acid deficiency during gestation in the rat can result in various effects ranging in severity from mild malformations to fetal death, depending on the duration of the deficiency period (1–3). It was therefore necessary first to establish the period of deficiency appropriate for pregnant guinea pigs. This was accomplished by subjecting adult animals, pregnant and non-pregnant, to a pantothenic acid-deficient diet. The effects of a transitory deficiency of the vitamin during pregnancy were then studied by measuring levels of pantothenic acid and fat in the livers of the offspring, as well as by standard observations of the young.

In a third phase of the work, changes in the liver fat and cholesterol of normal guinea pigs during development were examined to provide a firmer basis for interpreting the observations made in the experimental animals.

METHODS

Animals and diets. Young guinea pigs were purchased from commercial sources and were maintained with a pelleted puri-

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[2] A preliminary report of this research was presented at the Fifth International Congress on Nutrition, September, 1960, at Washington, D. C.

fied diet[3] which has been shown in our laboratory to support normal reproduction in this species (4). Ascorbic acid was given 3 times weekly by mouth in amounts to supply 15 mg/day.[4] α-Tocopherol in corn oil was fed 2 times each week by mouth, providing 1.5 mg/day for young animals and 3.7 mg daily for adult animals.

A pantothenic acid-deficient diet was prepared by omitting the vitamin from the pelleted purified ration.

To establish the length of time appropriate as a deficiency period in adult animals, 6 male guinea pigs, 7 non-pregnant females, and 3 pregnant females were transferred from the complete purified diet to the pantothenic acid-deficient ration. The animals were allowed to eat the ration ad libitum, and were given the diet until death occurred, or until the animals were killed when a moribund state was noted. The observation of soft, yellow livers in deficient animals led us to determine liver fat and moisture content. In some cases, liver pantothenic acid and coenzyme A values were also determined. Tissues from adult guinea pigs which received the complete purified diet throughout the experiment were used as controls. Pair-fed controls were examined to ascertain the influence of inanition on liver fat. In these cases, the food intake of the pair-fed control (receiving the complete purified diet) was limited to the amount of food eaten the previous day by the animal receiving the deficient diet.

The effect of pantothenic acid deficiency during gestation in the guinea pig was studied by transferring pregnant females, whose time of mating was known, from the complete purified diet to the pantothenic acid-deficient diet for one week at various periods of gestation. One week was chosen as the period of dietary deficiency on the basis of the experiments with adult animals described above. Pregnancies were carefully timed by the use of daily vaginal smears. Males were placed with the females only during the short period of estrus; mating was considered accomplished when sperm were observed in the vaginal smear. Since infertile matings are frequent in guinea pigs, it was necessary to verify pregnancy by palpation, but this was not possible until the embryos were large enough to be felt (about 30 days of gestation).

The females were allowed to deliver their young; some animals from each litter were killed at birth and others at 7 days of age. The animals were decapitated to avoid the effects of anesthetics on liver metabolism. Total pantothenic acid, moisture, and fat were determined in the livers of the young. In one case, a litter born to a female that received the deficient diet during the ninth week of pregnancy, 3 newborn that were found dead were used for tissue analysis. These animals had apparently died just before they were found; no autolysis was noted. Thirty-one females in which pregnancy was established were used in this experiment: 17 females used as controls received the complete purified diet throughout pregnancy; 14 females were given the same control diet except for a period of one week during pregnancy.

Liver fat concentrations during development were studied in offspring of guinea pigs receiving either the complete purified diet, or a stock diet. The stock diet consisted of a commercial guinea pig chow[5] supplemented with ascorbic acid as described above and with fresh greens. Liver cholesterol concentrations were studied in offspring of females fed stock diet. Animals were killed and liver analyses were made at ages ranging from 62 days of gestation to 21 days after birth.

Chemical analyses. Free and total pantothenic acid were determined by the method of Skeggs and Wright (5) after enzymatic release of the bound pantothenic acid by treatment with intestinal phosphatase and pigeon liver enzyme.

[3] The diet had the following percentage composition: vitamin-free casein, 30; cornstarch, 20; glucose, 10.6; sucrose, 10; wood pulp, 10; agar, 5; salts, 6; cottonseed oil, 5; potassium acetate, 2.5; magnesium oxide, 0.5; inositol, 0.2; and choline chloride, 0.2. Vitamins were added in amounts to provide for each kilogram of diet the following: (in milligrams) thiamine·HCl, 16; riboflavin, 16; pyridoxine·HCl, 16; Ca pantothenate, 40; nicotinic acid, 200; biotin, 1; folic acid, 10; 2-methyl-naphthoquinone, 5; p-aminobenzoic acid, 100; α-tocopherol, 100; vitamin B_{12}, 50; also vitamin A, 6000 IU; and vitamin D, 600 IU. The salts mix contained: (in grams) $CaCO_3$, 300; K_2HPO_4, 325; NaCl, 168; $FeSO_4 \cdot 7H_2O$, 25; $MgSO_4 \cdot 7H_2O$, 28; KI, 0.8; $ZnCO_3$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.3; $MnSO_4$, 2.3.

[4] We are indebted to Merck Sharp and Dohme, Inc., Rahway, New Jersey, and to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for supplies of vitamin B_{12} and ascorbic acid.

[5] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.

Preparation of enzyme solutions was according to Novelli et al. (6). Dowex treatment for removal of endogenous coenzyme A from pigeon-liver extract was used (7).

Coenzyme A was determined by the method of Kaplan and Lipmann (8). Liver fat was measured gravimetrically after extraction from the dried tissue with petroleum ether. Moisture was determined by drying at 60° to constant weight. The method of Sperry et al. (21) was used for the determination of cholesterol.

RESULTS

The effect of a pantothenic acid-deficient diet on survival of adult guinea pigs is summarized in table 1. The non-pregnant guinea pigs died within 10 to 41 days (mean, 25) when the vitamin was omitted from the diet. Three pregnant animals died within 9 to 16 days (mean, 13). At autopsy, most of the animals had hemorrhagic adrenals, and about one-fourth of them showed hemorrhages in the gastrointestinal tract.

The results of liver analyses in adult guinea pigs are summarized in table 2. The pantothenic acid-deficient diet produced a marked decrease in both pantothenic acid and coenzyme A levels in the liver. In contrast, the liver fat concentration was more than 10 times higher in the deficient animals than in the controls fed ad libitum (40% of the dry weight as compared with 3.3%). The pair-fed controls also, however, exhibited a high liver fat (37%); thus, the fatty livers of the deficient animals cannot be ascribed to a specific effect of pantothenic acid deficiency, since partial starvation alone could also produce the effect.

A comparison of food intake and body weight in pantothenic acid-deficient and

TABLE 1

Survival of adult guinea pigs fed pantothenic acid-deficient diet

Group	No.	Survival	
		Mean	Range
		days	*days*
Males	6	24.7	14–38
Non-pregnant females	7	24.3	10–41
Pregnant females	3	12.7	9–16

TABLE 2

Effect of pantothenic acid deficiency on liver analyses in adult guinea pigs (male and female)

Group	Pantothenic acid (total)		CoA		Fat		Moisture	
	Conc	No. samples	Conc	No. samples	Conc	No. samples	Conc	No. samples
	μg/g		*units/g*		*% dry wt*		*% fresh wt*	
Complete diet (ad libitum)	57 [1] (41–68)	3	75(68–79)	3	3.3 ± 0.5 [2]	6	71 ± 0.7	6
Pantothenate-deficient	29 (20–47)	5	37(24–57)	5	40 ±3.7	13	65 ± 1.9	13
Complete diet (pair-fed)	—	–	—	–	37 ±6.6	6	67 ± 2.1	6

[1] Mean, with range in parentheses.
[2] Mean ± SE.

pair-fed animals showed that food utilization was much better in pair-fed controls than in deficient guinea pigs. An example is shown in figure 1.

These results suggest that the adult guinea pig is highly susceptible to a dietary deficiency of pantothenic acid.

The effect of a transitory dietary deficiency of pantothenic acid on reproductive performance in this species is summarized in table 3. Of a total number of 17 females receiving the complete purified diet, eight either aborted or died before term. All of these abortions and deaths, however, occurred before the beginning of the ninth week of gestation, and three of the abortions occurred before the sixth week. Thus, the reproductive performance of the controls was somewhat better than that of the animals given the pantothenic acid-deficient diet during the tenth week of gestation (in which 4 litters with live young

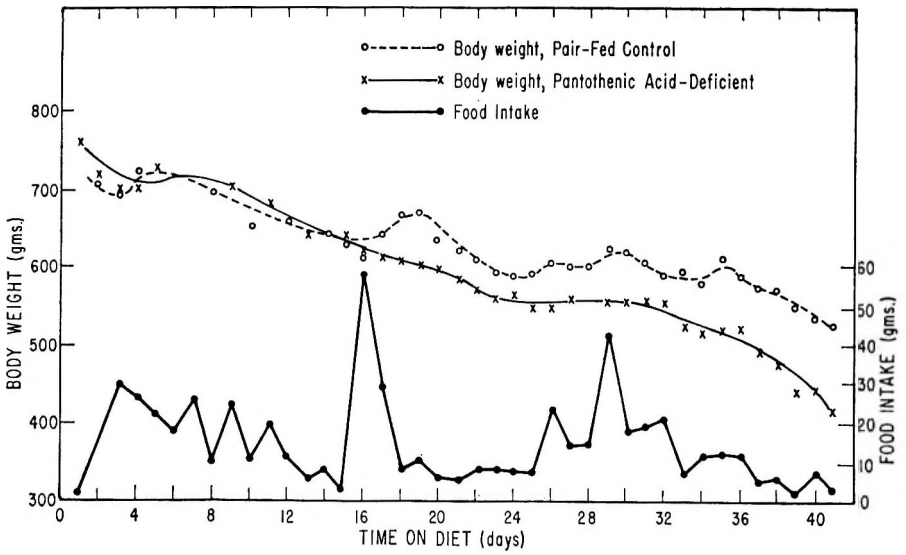

Fig. 1 Food intake and body weight in a pair-feeding experiment. The control animal received the amount of food eaten the previous day by the animal fed the pantothenic acid-deficient diet.

TABLE 3

Effect of a transitory dietary deficiency of pantothenic acid on reproductive performance of guinea pigs

Week of gestation in which deficient diet was fed	No. pregnant females	Reproductive performance				Offspring	
		No. with live young	No. with all young dead	No. aborted	No. died	Total no. born	No. born dead
		Controls					
	17 [1]	9	0	5	3		
	14 [2]	9	0	2	3	25	6
	9 [3]	9	0	0	0		
		Experimental					
10th	5	4	1	0	0	13	3
9th	6	1	2	2	1	7	4
7th	2	1	0	0	1	5	2
6th	1	1	0	0	0	3	0

[1] Total number.
[2] At beginning of sixth week of gestation.
[3] At beginning of ninth week of gestation.

were produced from 5 pregnancies), and was considerably better than that of the 6 females given the deficient diet during the ninth week of gestation. In the ninth-week group, only one litter with living young was produced, and two abortions and one death occurred. Thus, there were 9 control females who started the ninth week of pregnancy; all of these produced litters with living young. In the deficient group, however, there were 11 females who started the ninth week of pregnancy; of these, 5 produced litters with living young. No gross morphological abnormalities were noted in any of the offspring of deficient animals, but one showed behavioral disturbances (postural difficulties and some tremor) in the 6 days it lived.

The effect of transitory pantothenic acid deficiency during gestation upon liver fat and pantothenic acid levels in the offspring is shown in figure 2. The liver fat level of the normal newborn was found to be about 10 times higher than that of the adult, but by 7 days of age it had decreased almost to the adult value. In contrast, liver pantothenic acid concentration in the normal newborn was about 60% of the adult value, but increased during the first 7 days after birth.

A dietary deficiency of pantothenic acid during the tenth week of gestation (day 63 of gestation to birth) appeared to produce a significant increase in the liver fat of the newborn. (In 6 newborn controls, the range of values was 30 to 39% fat, whereas in 6 newborns whose mothers were given the deficient diet during the tenth week, the range was 36 to 65%.) At the same time, the liver pantothenic acid level of this group was lower than normal, both at birth and at 7 days of age.

Young whose mothers had received the deficient diet during the ninth week (56 to 63 days of gestation), the seventh week (42 to 49 days of gestation), or the sixth week (35 to 42 days of gestation) showed no significant changes in liver fat. In contrast, all of these groups had liver pantothenic acid levels at birth considerably lower than those of the control newborns, but in the seventh-week and sixth-week groups, concentration of the vitamin had reached the normal value by 7 days of age.

The results of the study on liver fat levels during development are shown in figure 3. Liver fat concentration at birth was more than 10 times the adult level. At 62 days of gestation it was even higher than at birth, but it decreased rapidly after parturition. By 2 days of age, the liver fat level was about one-third that of

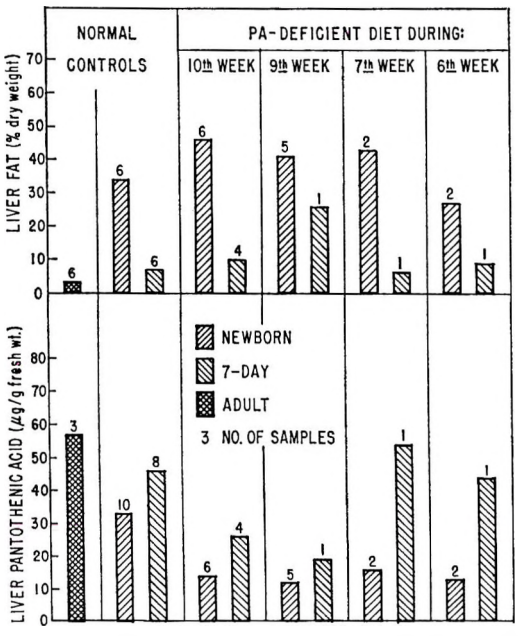

Fig. 2 Effect upon the offspring of a transitory dietary deficiency during gestation in guinea pigs. Each bar represents the mean of several samples, the number of which is shown above the bars.

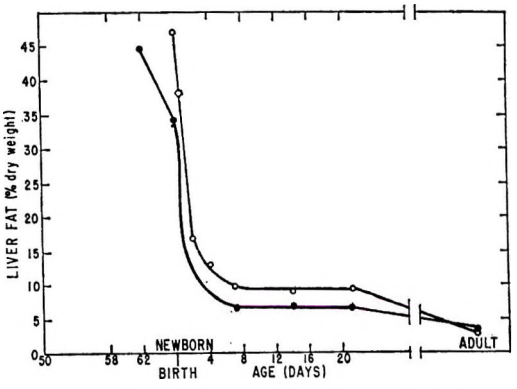

Fig. 3 Liver fat concentration in developing guinea pigs. Each point represents the mean of from 2 to 10 animals (in most cases, 5); each mean represents animals born in from 2 to 6 litters. Open circles indicate stock diet; closed circles, complete purified diet.

the newborn. It continued to decrease until 7 days of age, and then remained quite constant to 21 days, although it was still above the adult level.

In contrast, liver cholesterol (summarized in table 4) increased slightly from birth to 4 days of age, decreased slightly at 7 days, and then leveled off. Variation in liver cholesterol values was great; nevertheless, liver cholesterol levels in the neonatal period did not follow the same pattern as did liver fat.

DISCUSSION

The experiment on pantothenic acid deficiency in adult guinea pigs suggests that the adult of this species is highly susceptible to a dietary deficiency of the vitamin. The pregnant guinea pig was even more susceptible than the non-pregnant animal. Adult rats, even with the added requirements of pregnancy, when given a pantothenic acid-deficient diet show no ill effects after one month (3, 9), whereas adult non-pregnant guinea pigs died after a period of from 16 to 42 days. In the rat, pantothenic acid deficiency has a relatively small effect upon the maternal organism, even when the deficiency is severe enough to cause fetal death and resorption in the young (1–3).

The observation of high liver fat in pair-fed controls suggests that the fatty livers of pantothenic acid-deficient guinea pigs were the result of starvation. Nevertheless, the production of fatty livers in these animals is of interest. Guehring and coworkers (10) showed that pantothenic acid-deficient rats did not develop fatty livers even when fed a high cholesterol diet which greatly increased liver fat in rats receiving normal amounts of pantothenic acid. Other diets which also produced fatty livers in rats given pantothenic acid did not have this effect in pantothenic acid-deficient rats (11). There is thus a significant species difference in the response of liver lipids to pantothenic acid deficiency. The guinea pig, unlike the rat, is similar in this respect to the dog and the hamster which also develop fatty livers when fed a diet deficient in this vitamin (12, 13).

The results of the experiments concerning a transitory deficiency of pantothenic acid during gestation are in accord with observations on the changes in pantothenic acid and coenzyme A levels in fetal tissue. Hurley and Volkert (14) have recently shown that pantothenic acid and coenzyme A levels increased sharply in developing guinea pig liver beginning at 58 days of gestation, and reached a maximum at 4 days after birth. Thus, the dietary deficiency of pantothenic acid imposed during the ninth week (days 56 to 63) of gestation, at the time when the fetal liver was beginning to increase its accumulation of the vitamin, appeared to have a more detrimental effect on the young than the deficiency during the tenth week (day 63 of gestation to birth).

In one newborn, whose mother had been given the deficient diet from 56 to 63 days of gestation, behavioral abnormalities, as well as an extremely fatty liver, were observed. (Ershoff and Kruger (15) have observed a neurological defect in offspring of pantothenic acid-deficient rats.) This animal maintained its birth weight throughout its 6-day postnatal life; the high fat content of the liver could therefore not have been caused by inanition. The high

TABLE 4

Liver moisture and cholesterol in developing guinea pigs [1]

Age	No. samples	Moisture		Cholesterol	
		Range	Mean	Range	Mean
days		*% fresh wt*	*% fresh wt*	*% dry wt*	*% dry wt*
Newborn	5	49–69	62	0.43–1.29	0.92
2	3	53–74	64	0.93–3.31	1.83
4	4	73–77	74	1.51–2.51	1.85
7	5	71–81	74	1.11–1.76	1.55
14	5	64–93	75	1.01–4.74	1.95
21	4	72–87	77	1.30–3.99	2.08

[1] Fed stock diet.

liver fat might be viewed as a fetal condition which persisted into postnatal life, perhaps because of the absence of pantothenic acid during the critical period of gestation. In another litter, deficient from 63 days of gestation to birth, the young were born dead and had livers whose fat content was well above that of normal newborns. This fat accumulation also could not have been a result of inanition; during the period of deficiency, the mother appeared to be in good health and continued to gain weight satisfactorily.

These experiments suggest that the greatest need for pantothenic acid during fetal development of the guinea pig is in the period shortly before birth, possibly from about 58 days of gestation to term. It is of interest that nutritional deprivation coming at the very end of pregnancy should have a demonstrable effect upon the offspring. This is in contrast with many experiments on teratogenic effects of nutritional factors (16, 17). It is possible, however, that deficiencies of other nutrients may also be critical during the period close to term.

The fat content of the liver in the newborn guinea pig was strikingly higher than that of adult liver, and decreased rapidly after birth. These results are of interest in relation to the observation that coenzyme A levels in the liver increased maximally in the 4 days after birth. It is possible that the decrease in liver fat occurring in the neonatal period is related to the increasing level of coenzyme A during this period. This coenzyme is involved in both synthesis and breakdown of fat, however. It is therefore noteworthy that Hard et al. (18) have reported a sharp increase in the fetal liver fat of guinea pigs between 55 and 62 days of gestation, a period in which there is a rapid accumulation of coenzyme A (14).

The work of Goldwater and Stetten (19) showed that fetal liver fat and cholesterol were not derived from the maternal circulation, and Popjak (20) further concluded that there was no significant degradation of lipids by the fetus during intrauterine life. Popjak also noted a high liver fat content in the newborn rabbit.

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Mineral Balance Studies with the Baby Pig: Effects of Dietary Magnesium 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 magnesium of 75, 225, 325, 425 and 825 ppm. Dietary levels of Ca and P were maintained at 0.8 and 0.6%, respectively, with a constant dietary vitamin D_3 level of 1800 IU/kg. Food intake and consequent mineral intake were reduced in animals receiving 75 ppm dietary Mg. Increasing dietary Mg level above 225 ppm did not significantly affect Ca or P balance but the maximal percentage retention of Ca and P was obtained with pigs receiving 325 ppm of dietary Mg. Urinary Ca excretion was significantly increased by increased dietary Mg. Excreta Mg and Mg balance were both positively related to Mg intake. Maximal percentage Mg retention was obtained with dietary Mg levels of 225 or 325 ppm.

Studies by Hart and Steenbock (1) with adolescent pigs have shown that the addition of magnesium salts to a basal bran diet results in increased urinary calcium elimination and may result in a negative calcium balance. Studies by Bartley et al. (2), Freese (3) and Lenkeit and Freese (4) with baby pigs receiving a purified diet or milk diet have shown that magnesium balance is positively related to magnesium intake. Studies with other laboratory animals have generally shown adverse effects of increased dietary calcium and phosphorus upon dietary magnesium utilization (5), but increasing dietary magnesium has not generally greatly affected calcium and phosphorus utilization (6).

The present study was undertaken to determine the effects of dietary magnesium 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 magnesium requirement of the baby pig (7).

MATERIALS AND METHODS

Baby pigs used in this study were from the second of 2 trials conducted to determine their magnesium requirement (7) when fed purified casein-glucose diets. Calcium, phosphorus and magnesium balance studies were conducted with pigs receiving 75, 225, 325, 425 or 825 ppm. Dietary levels of Ca and P were 0.8 and 0.6%, respectively. Dietary sources of Ca, P and Mg were identical with those used in previous balance studies (8–10). Vitamin D_3 was supplied by cod liver oil[3] at a dietary concentration of 1800 IU/kg.

Twenty-nine mineral balance determinations were made when the baby pigs were 4 to 6 weeks of age. Methods of performing the balance trials were similar to those described previously (10). Three 3-day collections with controlled intakes were made on 2 animals receiving dietary Mg at each level with the exception that the final collection for one of the pigs receiving 75 ppm of dietary Mg was not completed because it died suddenly in Mg-deficiency tetany. A 3-day adjustment period with identical intake preceded each collection period. Distilled water served as the sole source of drinking water. Analyses of food, fecal and urine Ca, P and Mg were performed by methods indicated in a previous

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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).

[3] Standardized Cod Liver Oil (6.25 μg vitamin D_3/g), Parke, Davis and Company, Detroit.

study (10). Data were examined by analysis of variance, and statistical significance of treatment differences was determined by the multiple range test of Duncan (11). Correlation coefficients were determined for certain factors of mineral balance and tested for significance (12).

RESULTS AND DISCUSSION

The summarized data from the balance studies are presented in table 1. Both of the pigs receiving 75 ppm of dietary Mg had depressed appetites which necessitated reduced controlled food intakes and resulted in reduced mineral intakes. There was no depressing effect upon food intake of pigs receiving higher dietary Mg concentration, and identical near-maximal controlled intakes were maintained in simultaneous collections. Increasing the dietary Mg concentration above 225 ppm did not significantly affect fecal Ca or P, urine P, Ca or P balance, or Ca or P retention. Maximal Ca or P balance and the

TABLE 1

Daily calcium, phosphorus and magnesium excretion and retention as affected by dietary magnesium level

	Dietary Mg, ppm				
	75	225	325	425	825
No. of collections	5	6	6	6	6
Daily food intake, g	300 ± 33 [1]	433 ± 48[a]	433 ± 48[a]	433 ± 48[a]	433 ± 48[a]
Ca balance					
Daily Ca intake, g	2.40 ± 0.26	3.46 ± 0.38[a]	3.46 ± 0.38[a]	3.46 ± 0.38[a]	3.46 ± 0.38[a]
Daily fecal Ca, g	0.46 ± 0.08	0.43 ± 0.05	0.35 ± 0.06	0.47 ± 0.10	0.43 ± 0.10
Daily urinary Ca, g	0.002 ± 0.001	0.006 ± 0.001	0.014 ± 0.004	0.014 ± 0.001	0.041 ± 0.009[dd]
Daily Ca retention, g	1.94 ± 0.24	3.03 ± 0.35[a]	3.10 ± 0.34[a]	2.98 ± 0.33[a]	2.99 ± 0.33[a]
Ca retention, %	81 ± 3	87 ± 1	89 ± 2[a]	86 ± 2	86 ± 2
P balance					
Daily P intake, g	1.80 ± 0.20	2.60 ± 0.29[a]	2.60 ± 0.29[a]	2.60 ± 0.29[a]	2.60 ± 0.29[a]
Daily fecal P, g	0.22 ± 0.04	0.22 ± 0.03	0.20 ± 0.04	0.27 ± 0.05	0.28 ± 0.06
Daily urinary P, g	0.22 ± 0.05	0.32 ± 0.06	0.29 ± 0.04	0.33 ± 0.08	0.33 ± 0.07
Daily P retention, g	1.36 ± 0.14	2.06 ± 0.25[a]	2.11 ± 0.24[a]	2.00 ± 0.18[a]	1.99 ± 0.20[a]
P retention, %	76 ± 3	79 ± 2	81 ± 2	78 ± 3	77 ± 3
Mg balance					
Daily Mg intake, mg	23 ± 2	97 ± 13[a]	141 ± 16[aa]	186 ± 21[aa,b]	357 ± 39[dd]
Daily fecal Mg, mg	8 ± 1	21 ± 3	28 ± 5	50 ± 9[a]	145 ± 24[dd]
Daily urinary Mg, mg	0.4 ± 0.1	1.2 ± 0.4	4 ± 1	9 ± 1[bb,c]	20 ± 3[dd]
Daily Mg retention, mg	14 ± 2	75 ± 9[a]	109 ± 14[aa]	126 ± 14[aa]	192 ± 38[cc,d]
Mg retention, %	62 ± 3	77 ± 2[aa,b]	76 ± 5[aa,b]	68 ± 4[a]	52 ± 6

[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] $P < 0.01$.
[d] Significantly greater than all other values ($P < 0.05$); [dd] $P < 0.01$.

percentage Ca or P retention were obtained with pigs receiving 325 ppm of dietary Mg. Urine Ca excretion was significantly increased by increased dietary Mg intake with a correlation coefficient of 0.73 ($P < 0.01$). Serum Ca and Mg concentrations in these animals were positively related as were also serum Mg concentration and Mg intake (7). The increased serum Ca concentration present concomitantly with increased serum Mg concentration as a consequence of increased Mg intake could account for the increased urinary Ca without changing the secretory constant for renal clearance (13). The failure of dietary Mg level to affect Ca or P balance other than urinary Ca is illustrated in figures 1 and 2.

The data in table 1 indicate that increasing dietary Mg concentration results in both increased excreta Mg and increased Mg retention. This is in agreement with previous observations (2, 3) and is well illustrated in figure 3. This is further verified by the significant relationship between the factors of Mg intake, excretion and retention. The correlation coefficients of all animals for these factors were: Mg intake and Mg retention (0.91), Mg intake and excreta Mg (0.90), Mg intake and

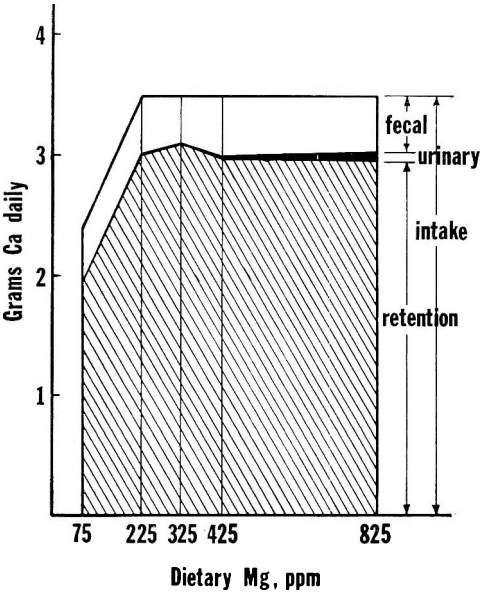

Fig. 1 Calcium balance as affected by dietary Mg level.

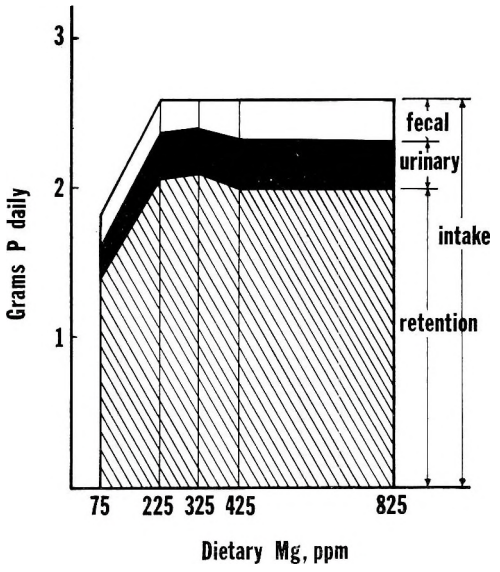

Fig. 2 Phosphorus balance as affected by dietary Mg level.

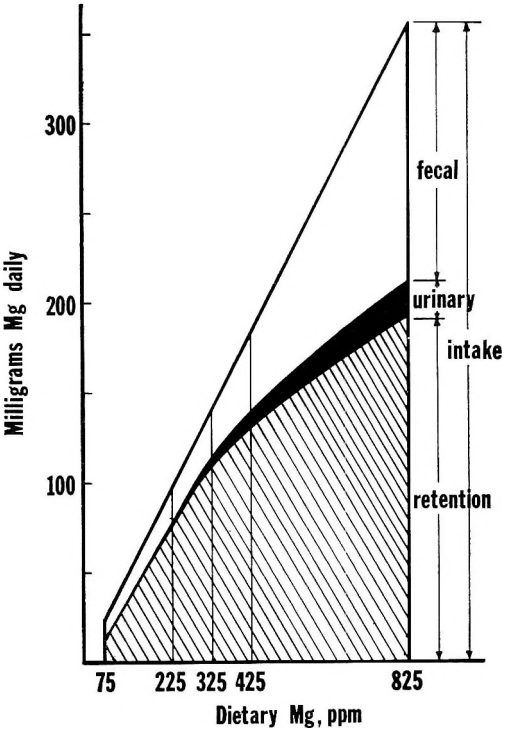

Fig. 3 Magnesium balance as affected by dietary Mg level.

fecal Mg (0.87), fecal Mg and urinary Mg (0.79), Mg intake and urinary Mg (0.75) and Mg retention and excreta Mg (0.61). All of these correlation coefficients were statistically significant ($P < 0.01$). When Mg intake and the percentage Mg retention of all animals were correlated the coefficient was -0.32 (nonsignificant); however, when values for the Mg-deficient pigs (75 ppm) were not considered in this correlation the coefficient became -0.53 and statistically significant ($P < 0.01$).

Pigs in this study (excluding those receiving dietary Mg at the lowest level) made an average body weight gain of 300 g daily during the course of the balance trials. Using the assumption of Bartley (2) that Mg concentration of the pig's body is 0.03%, the required daily Mg retention to maintain this body Mg concentration is 90 mg. Pigs receiving 325 ppm of dietary Mg retained 109 mg daily which appears to be an adequate margin of safety.

Data obtained in this study corroborate the work of Forbes (6) with the rat, in that increasing the level of dietary Mg did not significantly affect daily Ca or P retention but increased daily Mg retention. The balance data do not indicate a higher dietary Mg requirement than that of 325 ppm determined by other criteria (7).

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Value of Selenium in Alfalfa for the Prevention of Selenium Deficiencies in Chicks and Rats [1]

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ABSTRACT The value of the Se contained in alfalfa grown at a low level of available S, and in alfalfa grown at a high level of available S, was compared with that of Se from Na_2SeO_3 for the prevention of exudative diathesis in chicks and liver necrosis in rats. All of the sources of Se were supplied as additions to basal diets low in vitamin E and containing Torula yeast as the principal source of protein. The amount of alfalfa in the diets was held constant by using a low Se alfalfa in the diets containing Na_2SeO_3. Since the added alfalfa made up less than 2% of the total diet, levels of dietary S were nearly constant for all comparisons of Se sources. Se from the different sources, i.e., Na_2SeO_3, low S alfalfa and high S alfalfa, tended to be of similar value for the prevention of liver necrosis in rats and in the promotion of growth in both species. The Se in the high S alfalfa was about 25% less effective than the Se from the other sources for the prevention of exudative diathesis in chicks.

Several experimental and naturally occurring animal disease syndromes have been shown to respond to either dietary addition or subcutaneous injection of Se (1, 2). Much of the existing information on the value of dietary Se in the correction of these disease syndromes has been obtained by the addition of inorganic selenites to animal diets fed under experimental conditions (2–4). There is evidence that different Se compounds vary in their effectiveness in the prevention of liver necrosis in rats, and the existence of an especially effective form (Factor 3-Se) is postulated (5). Although some studies (6, 7) indicate that the Se contained in forage plants is at least partially effective in the correction of Se-responsive diseases of sheep and cattle, quantitative comparisons of the effectiveness of the Se in plants as compared with inorganic selenites are not available. This information is needed in order to utilize the results of experiments involving dietary additions of selenite in the diagnosis of "field" cases of Se deficiency where the Se contained in plants is the only dietary source of this element. Information on the biological value of Se in plants is also needed to evaluate the possibility of using Se treatment of the soil as a technique for the prevention of Se-responsive diseases in livestock.

These experiments were designed to determine the value of Se in alfalfa grown under controlled conditions of Se and S supply in the soil for the prevention of exudative diathesis in chicks and liver necrosis in rats.

EXPERIMENTS AND RESULTS

Production of alfalfa. The alfalfa used in these experiments was produced in 4 beds, 1.8 m × 4.9 m, 18.5 cm deep, constructed in a greenhouse. Each bed was lined with polyethylene sheeting and filled with a mixture of 30% Hudson silty clay loam and 70% washed quartz sand. The Hudson silty clay loam was taken from the top 14 cm of a Cornell University field. This field had been the source of hay used as a part of a ration that generally produced nutritional muscular dystrophy in lambs. The same uniform mixture of soil and sand was used in each bed.

The alfalfa (inoculated seed, variety Dupuit) was planted in rows about 18 cm apart. All beds received a uniformly heavy

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application of potassium, phosphorus, nitrogen, and boron, added as a solution of reagent grade salts. Additional K_2HPO_4 was added (in solution) uniformly to all beds periodically during the course of the experiment. The spaces between the rows of alfalfa were kept covered with strips of black polyethylene, except when additions of Se or S were being applied.

The alfalfa was allowed to become well established and was cut 3 times before any differential treatments were applied to the beds. Immediately after the third cutting, 1 g of Se was added, as a water solution of reagent grade Na_2SeO_3, to each of 2 beds selected at random. The addition of Se was made by pipetting the required amount of Se solution onto the surface of the soil-sand mixture along each midspace between alfalfa rows.

Following the addition of Se, the alfalfa was allowed to grow and was cut at the first appearance of blooms. Three cuttings of the alfalfa were made following the addition of Se. Immediately after the third cutting, S was added to two of the beds, one previously treated with Se, and one without Se treatment, at the rate of 50 g S/bed. The sulfur was added by pipetting a water solution of $MgSO_4$ onto the soil-sand mixture in each row midspace. An amount of Mg acetate containing the Mg equivalent to that in the $MgSO_4$ was added to the other 2 beds to eliminate any differences that might be due to differential levels of Mg in the S-treated and untreated beds. The addition of these S treatments resulted in one bed with no treatment (low Se–low S), one treated with Se only (high Se–low S), one treated with S only (low Se–high S), and one treated with both Se and S (high Se–high S).

Following the addition of the S, 3 additional cuttings were made for a total of 9 cuttings since planting, and the plant material was dried, ground, and stored in a cold room.

The alfalfa grew vigorously at all times, and there were no consistent or marked differences in yield among the 4 beds. The growth was quite comparable to that obtained under good growing conditions in the field. The addition of S resulted in a yield increase of about 3% over that of the beds without S, for the 3 cuttings taken after S treatment.

Analysis of the alfalfa. The Se content of the alfalfa produced was measured by a fluorometric method (8). The S content of the alfalfa was determined by X-ray fluorescence analysis, using a standard curve developed from analyses of forages by a chemical procedure.[2]

For the trials with chicks and rats, a composite of equal weights of the first and second cuttings following the application of S to the beds was utilized. The analyses of these composites are shown in table 1. The trichloroacetic acid (TCA)-insoluble Se was determined by shaking 0.5 g of the alfalfa with 50 ml of 5% TCA for 10 hours at room temperature, filtering, and washing the residue with distilled water. The Se content of the residue was then determined and reported as TCA-insoluble Se. Following dilution of the filtrate, an aliquot was treated with Na_2AsO_3 and hypo-phosphorus acid to precipitate $SeO_3^=$ and $SeO_4^=$ as elemental Se.

[2] Lazar, V. A., U. S. Plant, Soil and Nutrition Laboratory.

TABLE 1

Composition of alfalfa produced in the greenhouse with selenium and sulfur treatments of the soil

Sample	Soil treatment	Composition of alfalfa			
		Total sulfur	Total selenium	TCA-insoluble selenium	Inorganic selenium
		%	*ppm*	*ppm*	*ppm*
Low Se-low S	None	0.19	0.11	—	—
Low Se-high S	Sulfur	0.43	0.08	—	—
High Se-low S	Selenium	0.20	4.7 ±0.37 [1]	3.5	0.7
High Se-high S	Selenium + sulfur	0.48	4.6 ±0.49 [1]	3.0	0.8

[1] Mean ± SD.

The Se precipitated in this way was then redissolved in HNO_3, measured fluorometrically, and is shown as inorganic Se in table 1. There is a possibility that organic Se compounds may have been converted to inorganic Se during the precipitation of elemental Se.

Experiments with chicks. A preliminary experiment was conducted to ascertain whether it would be necessary to remove the vitamin E contained in the alfalfa in order to assay the value of the Se in the alfalfa. Low Se–low S, and high Se–low S alfalfas, each one with and without extraction of the alfalfa with absolute ethanol to remove vitamin E, were added to a basal ration known to result in a high incidence of exudative diathesis in chicks. The additions of alfalfa ranged from 0.15 to 0.6% of the diet. Male chicks of Cobb strain were used. The results of this preliminary experiment indicated that the Se in the alfalfa tended to protect chicks from exudative diathesis, and that it would not be necessary, as long as the additions of alfalfa to the diet were kept at a low level, to remove the vitamin E from the alfalfa to assay the value of Se in the alfalfa.

A second experiment was designed to provide a more accurate measurement of the value of Se contained in alfalfa for the prevention of exudative diathesis, and to determine the effect of the level of S nutrition of the plant upon the value of the Se contained in the plant.

The basal diet is shown in table 2, and the additions to this basal diet for achieving the desired levels and sources of Se are shown in table 3. The diets compounded as shown provided comparisons of sources and levels of Se without variation in the total amount of alfalfa added to the diet. Furthermore, the effect of the levels of S nutrition of the plant upon the value of the Se in the plant could be determined under conditions where the total S content of the entire diet was maintained essentially constant because of the low percentage of alfalfa in the diets.

Duplicate lots of 11 one-day-old chicks were assigned at random to each of the 10 treatments. The chicks were hatched from eggs produced by a group of hens maintained with a low vitamin E diet.[3]

TABLE 2

Composition of diet for chick experiment [1]

	%
Torula yeast [2]	58.5
Glucose monohydrate [3]	26.42
Stripped lard [4]	4.0
Cellulose	3.0
L-Arginine·HCl	0.2
Glycine	0.4
DL-Methionine	0.3
DL-Phenylalanine	0.1
Vitamin mix [5]	1.22
Mineral mix [6]	4.7
Ground alfalfa compounded as in table 3	1.16

[1] Trade names and company names are included for the benefit of the reader and do not infer any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.

[2] Lake States Yeast and Chemical Division, St. Regis Paper Company, Rhinelander, Wisconsin.

[3] Cerelose, Corn Products Company, Argo, Illinois.

[4] Distillation Products Industries, Rochester, New York.

[5] Vitamins in glucose, amount/kg of diet: 10,000 IU vitamin A; 1,500 ICU vitamin D_3; 2.44 mg menadione sodium bisulfite; 0.20 mg biotin; 20 mg Ca pantothenate; 4 mg folic acid; 50 mg niacin; 4.5 mg pyridoxine·HCl; 10 mg riboflavin; 10 mg thiamine·HCl; 20 μg vitamin B_{12}; 2.2 g choline chloride, 70% aqueous solution.

[6] Mineral mixture: (% of diet) 1.25 $CaHPO_4 \cdot 2H_2O$; 2.00 $CaCO_3$; 1.40 NaCl; 0.05 $MnSO_4 \cdot H_2O$; 0.00026 KI; 0.00017 $CoCl_2 \cdot 6H_2O$; 0.00083 $NaMoO_4 \cdot 2H_2O$.

Feed and water were supplied ad libitum, and the chicks were maintained with the basal diet without Se or alfalfa additions for 5 days before being fed the experimental diets. The chicks were maintained with the experimental treatments for 17 days. During this time, they were examined for exudative diathesis every other day.

The results of this experiment are shown in table 4. Analysis of variance of the data on the percentage of exudative diathesis indicated that the differences between the different levels of added Se were highly significant ($P < 0.01$). The inorganic selenite resulted in significantly less ($P < 0.05$) exudative diathesis than did similar amounts of Se contained in alfalfa, and chicks fed diets compounded with the low S alfalfa had significantly less ($P < 0.05$) exudative diathesis than did chicks fed diets compounded with high S alfalfa. However, at the 25- to 27-ppb level of added Se, there were fewer deaths among those chicks receiving Se from alfalfa than among those receiving Na_2SeO_3. The statistical significance of this difference in mortality was not evalu-

[3] Poultry Department, Cornell University.

TABLE 3

Se levels and sources in diets for chicks and method of compounding the Se additions

Treatment	Level of added Se	Material added to diet
	ppb	
C1	—	1.16% Low Se-low S alfalfa
C2	—	1.16% Low Se-high S alfalfa
C3	27	0.58% Low Se-low S alfalfa + 0.58% high Se-low S alfalfa
C4	27	0.58% Low Se-high S alfalfa + 0.58% high Se-high S alfalfa
C5	25	1.16% Low Se-low S alfalfa + Na_2SeO_3[1]
C6	25	1.16% Low Se-high S alfalfa + Na_2SeO_3[1]
C7	54	1.16% High Se-low S alfalfa
C8	54	1.16% High Se-high S alfalfa
C9	50	1.16% Low Se-low S alfalfa + Na_2SeO_3[1]
C10	50	1.16% Low Se-high S alfalfa + Na_2SeO_3[1]

[1] Added in water solution.

TABLE 4

Effects of different levels and sources of Se upon survival, exudative diathesis and growth of chicks

Treatment no.	Level of added Se	Source of Se	Level of S in alfalfa	Deaths	Exudative diathesis	Final wt [1]
	ppb			%	%	g
C1	—	—	low	78	100	218 ± 64
C2	—	—	high	64	100	160 ± 69
C3	27	Alfalfa	low	4	68	255 ± 72
C4	27	Alfalfa	high	0	78	243 ± 63
C5	25	Selenite	low	22	50	273 ± 45
C6	25	Selenite	high	14	64	264 ± 64
C7	54	Alfalfa	low	0	19	289 ± 31
C8	54	Alfalfa	high	0	36	285 ± 32
C9	50	Selenite	low	0	22	290 ± 30
C10	50	Selenite	high	0	22	292 ± 27

[1] Mean final weight ± SD of chicks surviving for the duration of the experiment.

ated. Analysis of variance of the data on final weights indicated that the differences between levels of Se were highly significant ($P < 0.01$), but that there were no significant differences in final weight due to sources of Se or to levels of S in the alfalfa used. All of these comparisons indicate that the different sources of Se tended to prevent exudative diathesis and to promote growth, and a general similarity in effectiveness of the different Se sources was evidenced.

Experiment with rats. An experiment, very similar in design to that conducted with chicks, was conducted to determine the value of the Se in alfalfa for protection of rats from liver necrosis. The diets for the rat experiment were compounded in the same manner as those for the chick experiment, except that the levels of added Se were 10 and 20 ppb instead of the 25- and 50-ppb levels used for chicks. The composition of the basal diet is shown in table 5, and the method of mixing high and low Se, high and low S alfalfa, and Na_2SeO_3 to achieve the desired levels and sources of Se is shown in table 6.

Ten weanling male rats (Holtzman) were allotted at random to each treatment (2 replications of 5 rats each) and kept in individual suspended wire-bottom cages. Feed and water were supplied ad libitum. All rats were maintained with the basal diet without addition of either alfalfa or Na_2SeO_3 for a 10-day period, at which time feeding of the treatments detailed in table 6 was started. The rats were checked 2 or 3 times each day, and any dead rats were examined immediately for gross evidence of liver necrosis. All the rats that died during the course of the experiment showed gross symptoms of liver necrosis.

TABLE 5

Diet for rat experiment

	%
Torula yeast [1]	30.0
Sucrose	57.06
Cellulose	3.0
Stripped lard [2]	4.0
Vitamin mixture [3]	1.5
Mineral mixture [4]	4.0
Alfalfa, compounded as shown in table 6	0.44

[1] Lake States Yeast and Chemical Division, St. Regis Paper Company, Rhinelander, Wisconsin.
[2] Distillation Products Industries, Rochester, New York.
[3] Vitamins in dextrose, amount per kg of diet: 13,500 IU vitamin A; 1,500 IU vitamin D; 675 mg ascorbic acid; 75 mg inositol; 1.125 g choline chloride; 33.75 mg menadione; 75 mg p-aminobenzoic acid; 67.5 mg niacin; 15 mg riboflavin; 15 mg pyridoxine·HCl; 15 mg thiamine·HCl; 45 mg Ca pantothenate; 0.3 mg biotin; 1.35 mg folic acid; and 20 μg vitamin B_{12}. (Nutritional Biochemicals Corporation, Cleveland.)
[4] Jones and Foster (9) plus cobalt chloride.

Feeding of the diets was continued for 48 days, at which time all surviving rats were examined for gross symptoms of liver necrosis. Only 3 slight cases of liver necrosis were noted among the surviving rats.

The results of this experiment are shown in table 7. The addition of 10 ppb Se to the diet provided relatively little protection from liver necrosis. The addition of 20 ppb Se to the diet, from all sources of Se, resulted in complete protection from liver necrosis and increased final weights of the rats. Because of the differences in numbers of rats surviving the experiment, an over-all analysis of variance for the entire experiment was not attempted. Analysis of variance of the final weights, considering only those

TABLE 6

Se levels and sources in the diets for rats, and the method of compounding the Se additions

Treatment	Level of added Se	Material added to diet
	ppb	
R1	—	0.44% Low Se-low S alfalfa
R2	—	0.44% Low Se-high S alfalfa
R3	10	0.22% Low Se-low S alfalfa + 0.22% high Se-low S alfalfa
R4	10	0.22% Low Se-high S alfalfa + 0.22% high Se-high S alfalfa
R5	10	0.44% Low Se-low S alfalfa + Na_2SeO_3[1]
R6	10	0.44% Low Se-high S alfalfa + Na_2SeO_3[1]
R7	20	0.44% High Se-low S alfalfa
R8	20	0.44% High Se-high S alfalfa
R9	20	0.44% Low Se-low S alfalfa + Na_2SeO_3[1]
R10	20	0.44% Low Se-high S alfalfa + Na_2SeO_3[1]

[1] Added in water solution.

TABLE 7

Effect of different levels and sources of Se upon percentage of death, survival time, and growth of rats

Treatment no.	Level of added Se	Source of Se	Level of S in alfalfa	Deaths	Survival time [1]	Final wt [2]
	ppb			%		*g*
R1	—	—	low	50	32.2 ± 95	142 ± 55
R2	—	—	high	90	30.8 ± 6.1	174
R3	10	Alfalfa	low	70	37.3 ± 10.0	193 ± 42
R4	10	Alfalfa	high	80	32.0 ± 4.1	163 ± 74
R5	10	Selenite	low	40	33.8 ± 2.6	188 ± 29
R6	10	Selenite	high	40	35.8 ± 4.5	187 ± 13
R7	20	Alfalfa	low	0	—	218 ± 26
R8	20	Alfalfa	high	0	—	202 ± 31
R9	20	Selenite	low	0	—	228 ± 26
R10	20	Selenite	high	0	—	213 ± 36

[1] Survival time is the mean number of days ± SD following the start of the experiment that the rats on each treatment lived. Rats surviving to the end of the experiment were not considered in this average.
[2] Mean final weight ± SD of rats surviving for the duration of the experiment.

treatments supplying 20 ppb Se, indicated that there were no significant differences in final weight due to the different sources of Se.

Se in rat livers. To determine the effects of some of the different treatments upon the concentration and total amount of Se in the livers of the rats, the livers from 4 randomly selected rats from several different treatments were analyzed for Se. The rats were selected from those surviving the feeding experiment. The livers were lyophylized, their dry weight was determined, and then ground to a fine powder. One-half gram of the dry powder was taken for Se determination.

The results of these Se measurements are shown in table 8. The concentration of Se in the liver and the total amount of liver Se was greater in rats receiving a diet containing 20 ppb Se than from those fed the diet without added Se. Se concentrations in the livers of rats receiving 20 ppb of dietary Se from alfalfa did not differ greatly from that in the livers of rats receiving equivalent Se from Na_2SeO_3.

DISCUSSION

The Se in both the low S and the high S alfalfa was of some value in preventing exudative diathesis in chicks and liver necrosis in rats, and in the promotion of growth in both species. For the prevention of exudative diathesis in chicks, Se from high S alfalfa was significantly less effective than Se from low S alfalfa, but the difference between the 2 sources of Se was not large. The Se in low S alfalfa could be considered to be about 25% more effective for the prevention of exudative diathesis in chicks than Se from the high S alfalfa. Other criteria for comparison of the value of the different Se sources did not indicate large or significant differences between Se sources, and their order of effectiveness tended to shift with the different criteria used. Considering the variability inherent in the types of assay experiments used, the similarity in the value of the different Se sources used is possibly the most striking of the results presented here.

The authors interpret these results as indicative that if either a particularly effective form of Se such as "Factor 3-Se" or a particularly ineffective form such as elemental Se (10) is present in alfalfa, it is present only as a minor part of the total Se. Furthermore, a marked increase in uptake by the plant of S from the soil brought about at most only a moderate shift of Se in the plant into compounds that are less effective in prevention of the Se-responsive diseases.

The similarity in value of Se from alfalfa as compared with equivalent amounts of Na_2SeO_3 is in agreement with the observations of Schwarz and Foltz (10) who reported similar effectiveness in prevention of liver necrosis for Na_2SeO_3, Na_2SeO_4, selenocystine, selenocystathionine, and selenomethionine, all of which are possible constituents of alfalfa. It is also in agreement with the work of Jones and Godwin (11) who demonstrated the assimilation of Se^{75} from alfalfa in mice.

The part (approximately 70%) of the Se in the alfalfa found to be insoluble in TCA is probably in the form of seleno amino acids combined into protein. A modest decrease in value of Se in alfalfa as compared with that in Na_2SeO_3 might be expected on the basis of incomplete digestion of the alfalfa protein by the

TABLE 8

Se in rat livers

Treatment no.	Level of Se in diet	Source of Se	Concentration of Se in liver [1]	Total liver Se
	ppb		*ppm dry basis*	*μg*
R1	0	—	0.11 ± 0.024	0.24
R7	20	High Se-low S alfalfa	0.16 ± 0.027	0.48
R8	20	High Se-high S alfalfa	0.21 ± 0.025	0.64
R9	20	Na_2SeO_3 + low Se-low S alfalfa	0.18 ± 0.014	0.44
R10	20	Na_2SeO_3 + low Se-high S alfalfa	0.20 ± 0.018	0.60

[1] Mean ± SD of 4 randomly selected rats for each treatment.

chick or rat, although the effects of different sources of Se upon the concentration of Se in the livers of rats indicate relatively uniform absorption of Se from the different sources.

It appears from these results that required levels of Se for animals, as determined in controlled experiments involving additions of selenites to animal diets, may be applied with some confidence to the diagnosis of "field" cases of Se-responsive diseases where the dietary Se comes from alfalfa. These conclusions will need to be confirmed using other plant species as carriers of the dietary Se.

It also appears, that, since the Se in the alfalfa plant is effective in the prevention of Se-responsive diseases, addition of Se to the soil might be a possible route toward prevention of these diseases, providing such addition could be made without danger of toxic concentrations of Se in the plants grown.

The effect of level of S nutrition of the plant upon the value of the Se in the plant, as investigated here, is only one of several possible points of interaction of S and Se in the soil-plant-animal system. Competitive effects of S and Se in biological systems have been reviewed by Schrift (12). There are also reports of an increased incidence of Se-responsive diseases when ruminants have been fed diets high in sulfates and low in Se (13, 14). It is entirely possible that high levels of S in animal diets may lead to an increased need for dietary Se, even though the Se contained in plants high in S is only slightly less effective than the Se contained in plants low in S, when fed under the conditions of nearly uniform levels of dietary S used in the experiments reported here.

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WESTERN HEMISPHERE NUTRITION CONGRESS

Organized by the Council on Foods and Nutrition

American Medical Association

November 8 – 11, 1965

Edgewater Beach Hotel, Chicago, Illinois

The inter-disciplinary congress will define current and future problems in nutrition and will determine the roles of medicine, agriculture, and technology in their solutions.

PROGRAM

Opening session

Nutrition in Medicine; The Physician's View of Nutritional Needs in the Western Hemisphere; Population and Food Supply — Implications in this Hemisphere; The Challenge to Technology.

Half-day sessions, including papers, forums and reviews

THE CHARACTERISTICS OF MALNUTRITION IN THE INFANT AND PRE-SCHOOL CHILD
or FOOD CONTROL AND REGULATIONS

THE NUTRITIONAL ECOLOGY OF THE INFANT AND PRE-SCHOOL CHILD

PROBLEMS IN FOOD PROTECTION AND TOXICOLOGY

NUTRITION IN MEDICAL AND PARAMEDICAL EDUCATION
or FOOD INDUSTRY TOURS

NUTRITION IN PUBLIC HEALTH

Closing session

MEETING THE CHALLENGES OF THE COMING DECADES
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It is anticipated that the Congress will attract scientists and educators from all parts of the Western Hemisphere. Every effort is being made to encourage participation from the many disciplines involved with problems in food and nutrition.

Total attendance will be limited to 1,000. A registration fee of $25 will include the Proceedings of the Congress and a banquet. All members of the American Institute of Nutrition will receive the published program. Additional information and requests for registration forms may be obtained by writing to the Department of Foods and Nutrition, American Medical Association, 535 North Dearborn Street, Chicago, Illinois 60610.