# Effects of Dietary Calcium upon Lipid Metabolism in Mature Male Rats Fed Beef Tallow [1]

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*ABSTRACT* In an attempt to elucidate the hypocholesterolemic and hypotriglyceridemic action of dietary calcium, 24 mature male Holtzman strain albino rats were fed a corn-soya ration containing 18% added beef tallow and 2% added cholesterol for 21 days. Dietary calcium was varied at 0.08, 0.2 and 1.2% of diet. Blood lipids decreased with increasing dietary calcium, the major decrease occurring at the 0.2% calcium level. Some tissue lipids decreased; none increased. Fecal lipids did not significantly increase at the 0.2% calcium level, but did at the 1.2% level. Fecal fatty acids became progressively more saturated with increasing calcium intake, but the preferential excretion of saturated fatty acids did not appear to be sufficient to alter significantly the blood fatty acids. Fecal bile acids significantly increased at the 0.2% calcium level, with no additional increase at the 1.2% calcium level. These results suggest that the lowering of blood cholesterol by increase in dietary calcium is mediated in part by increased excretion of bile acids.

Previous work in this laboratory has shown a decrease in serum cholesterol and triglycerides after increase in dietary calcium in man (1). Fecal lipid excretion also increased [6]; the literature on this has been reviewed by Yacowitz et al. (1).

The work reported here was carried out in an attempt to elucidate the possible mechanisms of this action of calcium.

## EXPERIMENTAL

Twenty-four male 400-day old albino rats of the Holtzman strain, mean weight 500 g, were divided into 3 groups and fed a corn-soya diet containing 18% added beef tallow and 2% added cholesterol (table 1). The diets contained 0.08, 0.2 and 1.2% calcium, respectively. Calcium was added to the diet as calcium carbonate and replaced an equal weight of washed sand so that all diets were kept isocaloric and equal in density. The diet was calculated to contain 15.63% protein, 20.31% fat, 1.18% fiber and 2.0% cholesterol. Assay results showed 0.35% phosphorus and 0.08% calcium. The fatty acid composition of the final diet is shown in table 2.

The rats were housed two in a cage and four cages were used per diet. Feces were collected daily, by cage, and assayed for lipids. After 21 days, the rats were anesthetized with sodium pentobarbital [7] and exsanguinated by heart puncture. Lipid analyses were performed on blood and tissues. Food was removed 18 hours prior to death, and the fasting time was comparable for all groups. The blood from the 2 rats in each cage was pooled in order to obtain sufficient sample for analysis. Entire tissues were washed free of blood with distilled water, ground with distilled water in a blender, lyophilized and assayed.

Lyophilized tissue lipids were extracted with chloroform:methanol (2:1) and assayed for the various lipids. The methods

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[6] Yacowitz, H., 1962 Effect of dietary calcium upon lipid metabolism in man. Federation Proc., *21*: 258 (abstract).

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TABLE 1
*Basal diet*

| | % |
|---|---|
| Ground yellow corn | 51.70 |
| Soybean oil meal, solvent process (44%) | 24.30 |
| Beef tallow, refined edible | 18.00 |
| Washed sand | 2.80 |
| Cholesterol, USP | 2.00 |
| Sodium chloride | 1.00 |
| DL-Methionine | 0.20 |
| Vitamins added/kg ration | |
| | *IU* |
| Vitamin A palmitate | 5500 |
| Vitamin E acetate | 44.60 |
| | *mg* |
| Choline | 330 |
| Butylated hydroxytoluene | 124.5 |
| Niacin | 27.5 |
| Riboflavin | 8.7 |
| Ca D-pantothenate | 8.7 |
| Vitamin K (menadione) | 2.2 |
| Thiamine | 1.3 |
| Pyridoxine | 1.1 |
| | *μg* |
| Folic acid | 659.9 |
| Vitamin $B_{12}$ | 13.1 |
| Minerals added/kg ration | |
| | *mg* |
| Iron, as red ferric oxide | 58.1 |
| Manganese, as manganese sulfate | 124.5 |
| Copper, as cupric sulfate·$5H_2O$ | 21.9 |
| Zinc, as zinc carbonate | 42.1 |
| Iodine, as potassium iodide | 1.5 |
| Cobalt, as cobaltous chloride·$6H_2O$ | 0.8 |

TABLE 2
*Fatty acid composition of basal ration*

| Fatty acid | Mole % of total fatty acids |
|---|---|
| 12:0 | 0.45 |
| 14:0 | 2.11 |
| 14:1 | 0.88 |
| 16:0 | 22.38 |
| 16:1 | 4.39 |
| 18:0 | 15.18 |
| 18:1 | 46.14 |
| 18:2 | 7.53 |
| 18:3 | 0.93 |
| Saturated fatty acids | 40.11 |
| Monoenoic fatty acids | 51.40 |
| Polyenoic fatty acids | 8.45 |
| P/S ratio | 0.21 |

for the assay of serum lipids have been described previously (1,2). Fecal bile acids were determined by the titrimetric procedure of Roscoe and Fahrenbach (3). The technique of Williams (4) was used for isolation of fecal lipids, and the total lipids were determined gravimetrically. Digitonin precipitation was required in the estimation of fecal 3-β-hydroxysterols, reported as cholesterol, because of interfering chromogens.

For fatty acid analysis, phospholipids were isolated on silicic acid columns with methanol after first eluting neutral lipids with chloroform and diethyl ether. Cholesterol esters and triglycerides were separated by thin-layer chromatography on silicic acid plates, using diethyl ether, hexane and glacial acetic acid (80:20:1) as the developing solvent. Non-esterified fatty acids were extracted by the method of Borgstrom (5). The preparation of methyl esters and the conditions employed in gas-liquid chromatography have been described previously (6).

## RESULTS

With the diets used, the weight gain in all groups was comparable (table 3), as was the mean feed consumption corrected for feed wastage. Fecal excretion (table 3) increased progressively with increasing dietary calcium. In table 3 are shown the changes in serum lipid values for the 3 diets. A decrease in all serum lipids is observed at the 0.2% calcium level. All serum lipids showed a further decrease at the 1.2% calcium level, but the decrease in phospholipids and total cholesterol was minor.

To determine whether changes in serum lipids were reflected in the tissue lipids, the analysis of liver, heart and lung tissues was undertaken (table 4). In liver, no significant changes were noted in total lipids or phospholipids. Cholesterol decreased 17% at the 0.2% calcium level with no further decrease at the 1.2% level. Triglycerides did not significantly decrease at the 0.2% level, but at the 1.2% level the decrease was significant ($P < 0.05$). No significant changes were noted in heart lipids, whereas lung exhibited a significant decrease in total lipids ($P < 0.05$) at the 0.2% calcium level with no significant additional changes occurring at the 1.2% calcium level.

Feces were examined in order to correlate changes in serum and tissue lipids with the rate of fecal lipid elimination (table 5). No significant difference in

TABLE 3

*Effects of dietary calcium on serum lipids, feed consumption, mean weight gain and feces weight*

| Calcium level in diet | 0.08% (1) | 0.2% (2) | 1.2% (3) | Decrease between (1) and (2) | Decrease between (2) and (3) |
|---|---|---|---|---|---|
| Lipids | | | | % | % |
| Total lipids, mg/100 ml | 456 ± 56.5 [1] | 319 ± 30.55 | 208 ± 18.38** | 30.0* | 34.9* |
| Phospholipids, mg/100 ml | 121 ± 4.47 | 89 ± 4.24 | 85 ± 5.66** | 26.5** | 4.5 |
| Total cholesterol, mg/100 ml | 125 ± 15.49 | 76 ± 5.27 | 68 ± 3.69* | 39.2* | 10.5 |
| Triglycerides, mg/100 ml | 117 ± 17.58 | 103 ± 3.94 | 93 ± 3.58* | 11.9 | 9.8 |
| Mean feed consumption, g/rat/day | 16.3 | 17.5 | 16.3 | | |
| Mean weight, start, g | 496 | 499 | 505 | | |
| Mean weight, final, g | 523 | 524 | 527 | | |
| Mean weight gain, g | 27 | 25 | 22 | | |
| Wet feces weight, g/rat/day | 6.67 | 8.57 | 10.57 | | |
| Dry feces weight, g/rat/day | 3.13 | 3.57 | 4.30 | | |

* Significant at $P < 0.05$. ** Significant at $P < 0.01$. [1] Mean ± SD.
[2] Significances computed by analysis of variance technique.

TABLE 4

*Tissue lipids in rats fed graded levels of calcium*

| Calcium level | 0.08% | 0.2% | 1.2% |
|---|---|---|---|
| | Liver, g/entire tissue | | |
| Total lipid | 2.261 ± 0.158 [1] | 2.166 ± 0.141 | 1.833 ± 0.158 |
| Phospholipid | 0.395 ± 0.014 | 0.451 ± 0.016 | 0.360 ± 0.021 |
| Total cholesterol | 0.626 ± 0.044 | 0.518 ± 0.012 | 0.513 ± 0.045 |
| Triglyceride | 0.606 ± 0.012 | 0.567 ± 0.010 | 0.394 ± 0.013 |
| Mean liver wt, g | 14.880 ± 1.72 | 16.538 ± 2.88 | 16.24 ± 1.32 |
| | Heart, mg/entire tissue | | |
| Total lipid | 39.7 ± 1.6 | 39.6 ± 1.2 | 40.6 ± 1.9 |
| Phospholipid | 28.6 ± 1.3 | 20.5 ± 1.6 | 23.8 ± 1.4 |
| Total cholesterol | 0.8 ± 0.2 | 0.8 ± 0.1 | 0.8 ± 0.1 |
| Triglyceride | 2.9 ± 0.2 | 2.8 ± 0.1 | 3.0 ± 0.2 |
| Mean heart wt, g | 1.55 ± 0.14 | 1.47 ± 0.01 | 1.49 ± 0.04 |
| | Lung, mg/entire tissue | | |
| Total lipid | 34.2 ± 2.4 | 23.9 ± 2.8 | 20.0 ± 2.5 |
| Phospholipid | 13.8 ± 2.0 | 9.7 ± 0.8 | 8.5 ± 0.4 |
| Total cholesterol | 2.8 ± 0.6 | 1.8 ± 0.1 | 2.9 ± 0.1 |
| Triglyceride | 7.0 ± 0.3 | 5.3 ± 0.8 | 3.7 ± 0.8 |
| Mean lung wt, g | 2.01 ± 0.08 | 1.99 ± 0.05 | 1.92 ± 0.11 |

[1] Mean ± SD.

fecal total lipid excretion was found between the 0.08% and the 0.2% calcium levels, whereas between the 0.2% and the 1.2% calcium levels, the fecal lipids significantly increased ($P < 0.01$). A significant increase was noted in digitonin precipitable, Lieberman-Burchard reacting substances, reported as cholesterol, at the 1.2% calcium level. As expected, there was an increase in lipid solvent extractable phosphorus, reported as phospholipids, with an increase in calcium ingestion.

An examination of fecal lipid fatty acids (table 5) showed a progressive increase in the degree of saturation of the fecal lipids with increasing calcium ingestion. There was an increase in the relative percentage of saturated fatty acids and a compensa-

TABLE 5

*Fecal lipid excretion in rats fed graded levels of calcium*

| Calcium level | 0.08% | 0.2% | 1.2% |
|---|---|---|---|
| | *mg/rat/day* | *mg/rat/day* | *mg/rat/day* |
| Total lipid | 477.2 ± 23.0 [1] | 624.8 ± 87.0 | 1112.7 ± 100.9** |
| Cholesterol [2] | 192.4 ± 26.2 | 213.9 ± 29.2 | 265.7 ± 34.5* |
| Phospholipid [3] | 15.6 ± 4.9 | 22.6 ± 4.2 | 25.0 ± 2.9* |
| | Fecal fatty acids, mole % of total fatty acids | | |
| Week 1 | | | |
| Saturated | 35.15 | 60.2 | 59.23 |
| Monoenoic | 24.82 | 25.18 | 28.94 |
| Di- and polyenoic | 39.19 | 11.94 | 18.14 |
| P/S | 1.27 | 0.20 | 0.33 |
| Week 2 | | | |
| Saturated | 48.48 | 54.83 | 62.48 |
| Monoenoic | 30.31 | 27.23 | 28.08 |
| Di- and polyenoic | 21.58 | 20.09 | 4.95 |
| P/S | 0.46 | 0.32 | 0.16 |
| Week 3 | | | |
| Saturated | 55.49 | 53.34 | 76.44 |
| Monoenoic | 26.11 | 25.73 | 20.22 |
| Di- and polyenoic | 17.23 | 14.45 | 3.31 |
| P/S | 0.32 | 0.25 | 0.05 |

[1] Mean ± SD.
[2] Digitonin precipitable, Lieberman-Burchard reacting material from a total lipid extract.
[3] Lipid solvent extractable phosphorus as phospholipid (lipid phosphorus × 25).
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

tory decrease in the relative percentage of dienoic and polyenoic fatty acids in the feces. This relationship continued at all calcium levels, and became more pronounced with the passage of time.

To determine whether the calcium-mediated preferential excretion of saturated fatty acids was of sufficient magnitude to alter the absorbed lipids, the fatty acids in the erythrocytes, cholesterol esters, triglycerides, phospholipids, free fatty acids and omentum were studied (table 6). A slight, non-significant increase was noted in erythrocyte fatty acid P/S ratio with increasing calcium. The P/S ratio of the serum free fatty acids also increased slightly with increasing calcium. Fleischman et al. (2, 7) reported that the serum free fatty acid P/S ratio would increase to above one within 72 hours in response to an unsaturated diet in man and would remain above one for the period of dietary adherence. An increase of this magnitude was not observed. Variable effects were noted in serum triglyceride fatty acids. No observable difference was noted in the serum cholesterol ester linoleate concentration at any of the 3 calcium levels. The serum phospholipid linoleate decreased somewhat with increasing calcium, as did the serum phospholipid P/S ratio. Examination of the omentum linoleate concentration indicated no observable change with calcium concentration. Thus the preferential fecal excretion of saturated fatty acids was insufficient to significantly alter the blood and omentum fatty acids during the period of study.

When the feces were examined for bile acids (table 7), an approximately threefold increase was observed consistently in the fecal bile acids at the 0.2% calcium level ($P < 0.001$) with no further increase at the 1.2% calcium level.

## DISCUSSION

Increased ingestion of calcium by rats fed a high fat, high cholesterol diet caused a significant decrease in serum total lipids, phospholipids and total cholesterol. The major decrease in serum total cholesterol occurred at the 0.2% calcium level, with a smaller additional decrease at the 1.2%

calcium level. This is in agreement with the previously reported human studies (1).

A determination of fecal lipids indicated that the major increase in fecal lipid excretion occurred at the 1.2% calcium level, whereas at the 0.2% calcium level, the fecal lipid change was not significant. Thus a large increase in fecal lipids is not a requisite for the calcium-mediated decrease in serum cholesterol. The measurement of cholesterol in the feces is actually a measurement of mixed 3-β-hydroxysterols, since digitonin precipitation and the Lieberman-Burchard reaction will not differentiate between cholesterol, sitosterol or coprostanol. More recently, Haust and Beveridge (8, 9) developed differential photometric procedures for the estimation of cholesterol and coprostanol, but these were not used in this work.

Tissue lipid analyses showed a decrease in liver cholesterol and triglycerides, lung total lipids and phospholipids. No changes were noted in heart lipids. In no case was there an increase in tissue lipids concomitant with a decrease in serum lipids, indicating that the decrease in serum lipids was not caused by deposition of these lipids in the tissues examined.

Examination of blood, omentum and fecal fatty acids showed that although the fecal fatty acids become progressively more saturated with increasing calcium concentration, this change was not significantly reflected by a concomitant increase in the degree of unsaturation of blood or omental fatty acids. It might be assumed that if calcium were to increase the excretion of saturated fatty acids, leading to a significantly increased absorption of unsaturated fatty acids, this increase in unsaturation would be reflected in the blood. The serum free fatty acids, which Fleischman et al. (2, 7) had shown to change rapidly with dietary manipulation, consequently should have become more unsaturated. Similarly, Horwitt et al. (10)

TABLE 6

*Effect of dietary calcium upon serum, erythrocyte and omentum fatty acids*

| Calcium level | 0.08% | 0.2% | 1.2% |
|---|---|---|---|
| | *mole % of total fatty acids* | | |
| Erythrocyte fatty acids | | | |
| Saturated | 58.20 | 58.16 | 57.93 |
| Monoenoic | 29.24 | 27.77 | 26.04 |
| Di-and polyenoic | 12.07 | 14.03 | 16.99 |
| Linoleate | 6.89 | 5.07 | 9.76 |
| P/S | 0.22 | 0.24 | 0.30 |
| Serum non-esterified fatty acids | | | |
| Saturated | 46.17 | 52.02 | 46.89 |
| Monoenoic | 40.34 | 27.85 | 32.90 |
| Di- and polyenoic | 13.45 | 20.05 | 20.14 |
| Linoleate | 13.45 | 12.15 | 14.17 |
| P/S | 0.29 | 0.39 | 0.43 |
| Serum phospholipid fatty acids | | | |
| Saturated | 50.00 | 51.61 | 47.48 |
| Monoenoic | 26.94 | 28.72 | 38.26 |
| Di- and polyenoic | 23.01 | 19.11 | 14.21 |
| Linoleate | 11.81 | 11.47 | 8.78 |
| P/S | 0.47 | 0.38 | 0.31 |
| Serum cholesterol ester fatty acids | | | |
| Saturated | 24.33 | 24.23 | 28.09 |
| Monoenoic | 54.08 | 43.89 | 37.96 |
| Di- and polyenoic | 23.60 | 31.86 | 32.84 |
| Linoleate | 8.94 | 9.78 | 8.80 |
| P/S | 0.97 | 1.32 | 1.42 |
| Serum triglyceride fatty acids | | | |
| Saturated | 64.57 | 49.37 | 51.32 |
| Monoenoic | 26.37 | 36.57 | 28.14 |
| Di- and polyenoic | 10.34 | 19.01 | 17.14 |
| Linoleate | 8.14 | 7.06 | 14.99 |
| P/S | 0.16 | 0.38 | 0.33 |
| Omentum fatty acids | | | |
| Saturated | 35.57 | 33.17 | 32.68 |
| Monoenoic | 48.03 | 47.71 | 54.09 |
| Di- and polyenoic | 17.61 | 17.11 | 14.68 |
| Linoleate | 16.28 | 12.55 | 12.90 |
| P/S | 0.50 | 0.52 | 0.43 |

TABLE 7

*Fecal bile acids in rats fed graded levels of calcium*

| Calcium level | 0.08% | 0.2% | 1.2% |
|---|---|---|---|
| | *mg/rat/day* | *mg/rat/day* | *mg/rat/day* |
| Week 1 [1] | 7.44 | 22.36 | 22.48 |
| Week 2 [1] | 9.13 | 22.19 | 20.38 |
| Week 3 [1] | 6.56 | 25.62 | 25.31 |
| Mean ± SD | 7.71 ± 0.24 | 23.39 ± 1.33 | 22.06 ± 1.81 |
| *P* | | < 0.001 | < 0.001 |

[1] Mean values.

showed that increasing the degree of unsaturation of the diet increased the degree of unsaturation of both human erythrocytes and chick cerebella. Similar results were shown by Day et al. (11) for cholesterol esters synthesized by rat reticuloendothelial cells. Since calcium did not increase the degree of unsaturation of serum and omental lipids, it thus appears that the action of calcium is not due to a significant alteration in the degree of unsaturation of absorbed fatty acids, but rather that the calcium acts through another mechanism to reduce lipids.

The threefold increase in the fecal excretion of bile acids provides a possible mechanism for the hypocholesterolemic action of calcium. The formation of calcium cholanates and their subsequent excretion in the stool could lower the cholesterol pool by increasing the excretion of bile acid, an oxidation product of cholesterol. This in turn could lower serum and tissue cholesterol. Since liver is the site of oxidation of cholesterol to bile acids, the 17% decrease in liver cholesterol observed at the 0.2% calcium level, the same calcium level at which the threefold increase in fecal bile acids was noted, gives added evidence for this mechanism. This calcium level was also the level at which serum cholesterol showed the greatest percentage decrease.

The exact mechanism by which calcium combines with bile acids, the site of the combination, the long-term effects of elevated calcium ingestion of a high fat, high cholesterol diet, and the effects of calcium in the presence of different types of fats, still require clarification. These aspects of the problem are currently under investigation.

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# Effect of Intestinal Damage Produced by *Eimeria necatrix* Infection in Chicks upon Absorption of Orally Administered Zinc-65 [1,2]

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*ABSTRACT* To study the effects of damage to the middle area of the intestine upon nutrient absorption, 3 trials were conducted in which the absorption of orally administered $^{65}Zn$ into the blood stream was determined in birds infected with *Eimeria necatrix*. Absorption rates were followed from one day post-inoculation until 35 days post-inoculation. In chicks severely infected with coccidiosis, absorption rates exceeded those of uninfected birds on the first day post-infection, decreased on the third day, decreased severely by the sixth day, and afterward began to return to levels observed in uninfected birds. By 21 days post-inoculation, absorption rates were near control levels, and at 28 and 35 days post-inoculation, absorption rates were much greater than control levels. Mild intestinal damage with slight inflammation was found to increase absorption, whereas severe damage and hemorrhaging decreased or stopped absorption. This absorption pattern explains the observations, previously reported, of nutrient deficiency symptoms appearing in chicks early in a coccidial attack, and of increased growth rates occurring during the recovery phase of the disease.

Few direct measurements have been made of the effects of intestinal disease upon the absorption of nutrients. Coccidiosis in chickens appears to provide an excellent situation for studying these effects, since pure cultures of various coccidial species attack rather specific areas of the gastrointestinal tract.

Stafseth (1) noted that rickets appeared or was more severe in birds suffering from coccidiosis. Erasmus et al. (2) have shown decreased liver storage of vitamin A in chicks suffering from coccidiosis caused by a combination of *Eimeria tenella* and *Eimeria acervulina*. This effect was particularly marked one week after administration of the oöcysts. These reports appear to indicate that absorption is impaired by the disease.

Several groups (3–5) have shown a period of increased growth rate during recovery from coccidiosis. Panda and Combs (5) suggest that this increase in growth rate is due to increased weight of the glands that respond to stress by hypertrophy. These observations might also be explained by a period of increased absorption, associated with the coccidial cycle or by increased food intake.

Several experiments were undertaken in our laboratory to determine the effects of infection of the intestinal protozoan *Eimeria necatrix* upon absorption of the trace mineral zinc. *E. necatrix* infections result in damage to the wall and musculature of the mid-portion of the small intestine. These trials were designed to follow absorption rates throughout the coccidial disease cycle in order to clarify, and perhaps resolve, the apparent conflict in the literature.

## PROCEDURE

In each of the 3 trials reported, broiler-type male chicks were raised to 4 weeks of age in electrically heated battery brooders with ad libitum access to a starter ration and tap water. At 4 weeks of age, the birds were divided at random into 2 groups in the first trial and into 3 groups in the second and third trials. One group in each trial was left uninoculated while the second group in trial 1 and one group each

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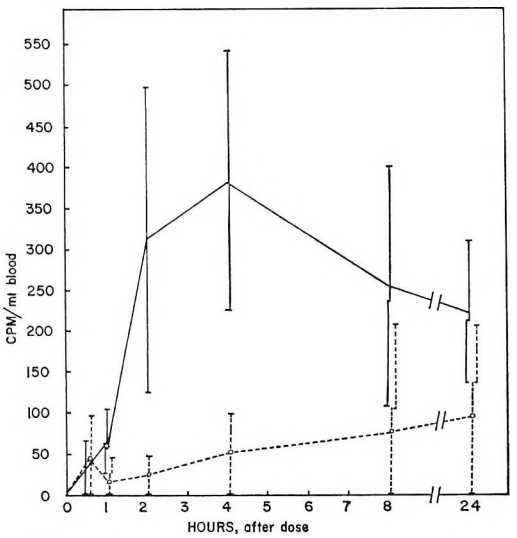


Fig. 1 Effect of severe *Eimeria necatrix* infection upon $^{65}Zn$ absorption 6 days after inoculation. Vertical bars indicate standard deviation of means. ——x—— uninfected controls; · · · ○ · · · *E. necatrix* infected.

in the second and third trials were given a single dose of 1 ml of an aqueous suspension of 65,000 sporulated oöcysts of *E. necatrix*. The remaining group in trials 2 and 3 was given 0.5 ml of the same oöcyst suspension or approximately 33,000 oöcysts. The number of sporulated oöcysts in the original suspension was determined with the aid of a hemocytometer. Dilution to the desired concentration was made with tap water.

At intervals after inoculation, as shown in tables 1, 2 and 3, 5 birds from the control group and from each of the infected groups were selected at random, weighed, placed in individual metabolism cages, and given a no. 4 gelatin capsule containing 50 μc of $^{65}Zn$ chloride absorbed on sucrose. Feed and water were available to each bird throughout the trial. At intervals of 0.5, 1, 2, 4 and 8 hours after administration of the tracer, one milliliter of blood was drawn from each bird by cardiac puncture and the amount of radioactivity was determined in a well-type scintillation counter with a pulse height analyzer. In the third trial, an additional blood collection was made 24 hours after tracer administration. At the end of the tracer period, all birds were killed and examined for intestinal damage. A visual scoring system was used in which zero denoted a normal intestine and 4 denoted a severely ballooned and hemorrhagic intestine, with the intermediate numbers signifying intermediate levels of damage. Weights and intestinal scores shown in the tables are those of the birds used in the tracer trial at that particular interval.

## RESULTS

In all trials, substantial amounts of the $^{65}Zn$ were absorbed by the control birds. Approximately 6% of the administered dose was in the blood by 4 hours after administration of the oral dose, assuming a blood volume of 10% of the body weight (6). Figure 1 compares the $^{65}Zn$ uptake curves obtained in uninfected birds and in severely infected birds, and indicates the range of blood radioactivity usually encountered.

In the first trial (table 1) impairment of absorption was evident at 3 days post-inoculation, particularly in those time intervals immediately after tracer administration. By 8 hours after $^{65}Zn$ administration, blood levels of the tracer more closely approached those of the uninfected birds, but absorption was still impaired. Some intestinal damage was apparent in the infected birds. At no time during any experiment was intestinal damage observed in the uninfected birds. Six days after oöcyst administration, absorption was severely impaired. Blood tracer levels recorded in infected birds from 30 minutes to 4 hours after the tracer administration were only 1 to 2% of those observed in uninfected control birds. At 8 hours post-inoculation, blood tracer levels were still only 17% of the control level. Intestinal damage at this time was severe, blood was observed in the droppings, and 23% of the infected birds had died.

By 12 days post-inocluation, the chicks had begun to recover from the infection. Absorption of the tracer was still slower in the infected birds than in the uninfected controls, but by 8 hours after tracer administration, tracer level in the blood of the infected chicks had reached, and slightly exceeded, those of the controls. The damaged intestines were healing in these birds, and they were gaining weight.

TABLE 1

*Effect of Eimeria necatrix infections on absorption of orally administered $^{65}Zn$ (trial 1)*

| Days [1] | Tracer in blood [2] | | | | | Intestinal score [3] | Average weight [4] | |
|---|---|---|---|---|---|---|---|---|
| | 0.5 hr | 1 hr | 2 hr | 4 hr | 8 hr | | Infected | Control |
| | % of control | | | | | | g | g |
| 3 | 43** | 35** | 46** | 90 | 75** | 1.4 | 680 | 686 |
| 6 | 2** | 1** | 1** | 2** | 17** | 3.6 | 414 | 703 |
| 12 | 18** | 30** | 67** | 87 | 115 | 2.4 | 602 | 964 |
| 14 | 95 | 74 | 86 | 116 | 134* | 1.8 | 705 | 955 |
| 21 | 55* | 48** | 75* | 121* | 195** | 1.0 | 955 | 835 |
| 28 | 50 | 78 | 67 | 84 | 211** | 0 | 1130 | 1241 |

[1] Infected birds were given 65,000 sporulated oöcysts of *E. necatrix* at day zero.
[2] Blood sample drawn at indicated times after oral administration of 50 μc $^{65}Zn$ on day indicated.
[3] 0 = normal undamaged intestine; 4 = severely damaged, hemorrhaged, ballooned intestine. Average of 5 birds.
[4] Average weights of 5 infected birds and 5 control birds used in tracer study on the day indicated.
* Significantly different from control ($P < 0.05$).
** Highly significantly different from control ($P < 0.01$).

TABLE 2

*Effect of Eimeria necatrix infections on absorption of orally administered $^{65}Zn$*

| Days [1] | Inoculation | Tracer in blood [2] | | | | | Intestinal score [3] | Average weight [4] | |
|---|---|---|---|---|---|---|---|---|---|
| | No. oöcysts | 0.5 hr | 1 hr | 2 hr | 4 hr | 8 hr | | Infected | Control |
| | | % of control | | | | | | g | g |
| 1 | 33,000 | 2[a]** | 3[a]** | 17[a]** | 91 | 153[a]** | 0 | 476 | 471 |
| | 65,000 | 97 | 108 | 153[a]** | 153[a]** | 127[a]** | 0 | 434 | |
| 3 | 33,000 | 47[b]** | 40[a]** | 55[b]** | 82[a]* | 104 | 0.2 | 545 | 573 |
| | 65,000 | 36[b]** | 69[a]** | 67[b]** | 153[a]** | 129[a]** | 0 | 537 | |
| 6 | 33,000 | 50[a]* | 68[a]** | 155 | 92 | 165[b]** | 2.0 | 502 | 612 |
| | 65,000 | 10[a]** | 15[a]** | 115 | 87 | 177[b]** | 2.6 | 555 | |
| 10 | 33,000 | 65[b]** | 60[b]** | 77[c]* | 73[b]* | 114 | 1.4 | 681 | 780 |
| | 65,000 | 48[b]** | 54[b]** | 44[a]** | 53[b]** | 50[a]** | 1.6 | 620 | |
| 14 | 33,000 | 12[a]** | 54[a]* | 44[a]** | 52[b]** | 58[b]** | 0.6 | 727 | 853 |
| | 65,000 | 96[c]** | 89[c]** | 63[b]* | 48[b]** | 69[b]** | 1.0 | 862 | |
| 21 | 33,000 | 29 | 37[b]** | 45[b]** | 113 | 42[a]** | 0.8 | 920 | 1141 |
| | 65,000 | 18 | 27[b]** | 62[b]* | 172[b]** | 98 | 0.8 | 899 | |
| 28 | 33,000 | 446[a]** | 196[a]** | 143[a]** | 191[a]** | 132[b]** | 0.8 | 1156 | 1375 |
| | 65,000 | 18 | 18[a]** | 16[a]** | 146[a]** | 145[b]** | 0.2 | 1124 | |

[1] Infected birds were given oöcysts orally at day zero.
[2] Blood sample drawn at indicated times after oral administration of 50 μc $^{65}Zn$ on day indicated.
[3] 0 = normal, undamaged intestine; 4 = severely damaged, hemorrhaged, ballooned intestine.
[4] Average weights of the 5 infected and 5 control birds used in tracer studies on day indicated.
[a] Different from control and other level of infection.
[b] Different from control only.
[c] Different from other level of infection only.
* $P < 0.05$.
** $P < 0.01$.

At 14, 21 and 28 days post-inoculation, absorption rate was still retarded, but by 4 to 8 hours after tracer administration, blood tracer levels exceeded those of the uninfected controls.

In the second trial, where 2 levels of coccidial infection were used, results varied somewhat from those of the first trial. Even though 65,000 sporulated oöcysts were administred to the more severely infected group, the level of infection produced was less than in the first trial as shown by the intestinal scores and weight data shown in table 2.

One day after inoculation, absorption was greatly impaired for the first 2 hours after nutrient administration in the birds given 33,000 oöcysts. After 2 hours in the moderately infected birds, and at all time intervals in the birds given 65,000 oöcysts, absorption levels were the same as, or slightly above, control levels. No intestinal

damage was observed in these birds. By 3 days post-inoculation, absorption followed the pattern observed in the first trial with little difference shown between levels of infection. Slight intestinal damage was noted in birds inoculated with 33,000 oöcysts each, but none in the more heavily inoculated birds. Weight gains were suppressed in both groups.

On the sixth day post-inoculation, absorption was severely impaired in both infected groups, but the absorptive impairment was graded according to inoculation level. Unlike the first trial where intestinal damage was severe, damage here was milder and absorption, although delayed, finally reached levels well above those of the controls.

As healing of the intestine progressed on the tenth through the twenty-eighth days post-inoculation, absorption rates tended to parallel healing rates in the severely infected groups. In the moderately infected groups, a period of poor absorption was noted at the 14- and 21-day observations and a period of greatly enhanced absorption occurred at 28 days post-inoculation. Absorption in the birds inoculated with 65,000 oöcysts was delayed, but by 4 hours after administration, blood tracer levels had increased above the levels observed in the uninfected controls.

In the third trial, an additional observation at 35 days post-inoculation and an additional determination of blood tracer levels at 24 hours post-administration of tracer were made. Two levels of oöcyst inoculations were used as in the second trial, but in this trial much more severe infections resulted as shown by the intestinal scores and weight data (table 3). At both levels of infection, absorption of tracer was increased at one day post-inoculation when compared with that of the uninfected controls. Retention of the tracer after 24 hours was greater in the infected birds, but this difference was not significant. By the third day post-inoculation, the absorption and retention of the tracer by the moderately infected birds

TABLE 3

*Effect of Eimeria necatrix infections on absorption of orally administered $^{65}Zn$*

| Days [1] | Inoculation | Amount of tracer in blood [2] | | | | | | Intestinal score [3] | Average weight [4] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. oöcysts | 0.5 hr | 1 hr | 2 hr | 4 hr | 8 hr | 24 hr | | Infected | Control |
| | | % of control | | | | | | | g | g |
| 1 | 33,000 | 221[b]** | 136[a]** | 150[b]** | 73 | 84 | 146 | 0 | 656 | 661 |
| | 65,000 | 193[b]** | 238[a]** | 172[b]** | 111 | 119 | 144 | 0 | 685 | |
| 3 | 33,000 | 76[b]* | 120 | 81 | 139 | 195[a]** | 226[b]* | 0.4 | 756 | 689 |
| | 65,000 | 41[a]** | 116 | 67 | 72 | 102 | 103 | 1.0 | 716 | |
| 6 | 33,000 | 7[b]* | 26[b]* | 13[b]** | 24[b]** | 19[b]** | 45[b]** | 3.4 | 756 | 758 |
| | 65,000 | 35[b]* | 19[b]* | 7[b]** | 7[b]** | 20[b]** | 43[b]** | 3.6 | 727 | |
| 9 | 33,000 | 44 | 19[b]** | 34[b]** | 18[b]** | 54 | 68 | 2.6 | 582 | 910 |
| | 65,000 | 43 | 32[b]** | 48[b]* | 30[b]** | 53 | 68 | 3.0 | 564 | |
| 14 | 33,000 | 62[a]** | 68[a]** | 66[a]** | 59 | 68 | 86 | 1.4 | 766 | 944 |
| | 65,000 | 4[a]** | 8[a]** | 15[a]** | 26[b]* | 37[b]* | 66 | 2.8 | 867 | |
| 21 | 33,000 | 4[b]** | 19[b]** | 144 | 63 | 120 | 118 | 0.8 | 1224 | 1274 |
| | 65,000 | 10[b]** | 17[b]** | 48 | 26[b]* | 25 | 39 | 1.6 | 1125 | |
| 28 | 33,000 | 2300[b]* | 1120[b]* | 385[b]** | 199 | 258 | 96 | 0.8 | 1435 | 1669 |
| | 65,000 | 278 | 228 | 83 | 67 | 135 | 54 | 1.4 | 1381 | |
| 35 | 33,000 | 1400[b]* | 381 | 288[b]* | 104 | 95 | 100 | 0.6 | 1566 | 1908 |
| | 65,000 | 1085[b]* | 251 | 285[b]* | 134[b]* | 125[b]* | 108 | 0.8 | 1485 | |

[1] Infected birds were given oöcysts orally at day zero.
[2] Blood sample drawn at indicated times after oral administration of 50 $\mu c$ $^{65}Zn$ on day indicated.
[3] 0 = normal, undamaged intestine; 4 = severely damaged, hemorrhaged, ballooned intestine.
[4] Average weights of the 5 infected and 5 control birds used in tracer studies on day indicated.
[a] Different from both control and other level of infection.
[b] Different from control only.
[c] Different from other level of infection only.
* $P < 0.05$.
** $P < 0.01$.

still exceeded that of the controls, whereas that of the more severely affected birds was about the same as that of the controls. Some intestinal damage was observed in the moderately infected birds and more damage was observed in the more severely infected birds.

On the sixth day, absorption was delayed and decreased in both groups of infected birds. Intestinal damage was severe and weight gains were greatly depressed in both infected groups when compared with those of the uninfected controls.

During the recovery period, absorption rates approached those of the uninfected birds and then greatly exceeded them. In the moderately infected group, absorption on the twenty-first day post-inoculation was delayed, but total absorption 2 hours after tracer administration had reached that of the uninfected chicks. After this, absorption in this group was more rapid and in greater quantity than in the control group through the thirty-fifth day post-inoculation. In the severely infected group absorption rates did not approach those of control until the twenty-eighth day, but then greatly exceeded them on the thirty-fifth day. Twenty-four-hour retention of tracer did not reach control levels until the thirty-fifth day post-inoculation.

In the first trial highly significant differences ($P < 0.01$) were found due to the effects of disease treatment, day of infection and time after administration of the tracer. Highly significant interactions were found between disease treatment and day of infection, disease treatment and time after tracer administration, and between time after tracer administration, disease treatment and day of infection. In the second trial all of the above differences and interactions were again highly significant except that the differences due to disease treatment were significant ($P < 0.05$). In the third trial, all effects and interactions were again highly significant except that the differences due to disease treatment and the interaction between disease treatment and day of infection approached significance.

## DISCUSSION

These data suggest that several factors affect absorption of nutrients from intestines damaged by disease. Passage rates of nutrients through the intestine and the amount of damage to the cells in the areas of the intestine where absorption occurs are two such factors that might be involved. *E. necatrix* infections slow intestinal passage rates, particularly during the fifth to tenth days post-inoculation (7). This might be expected to delay absorption, as was observed in this study, but it might also increase later absorption since the nutrients would be in contact with the intestinal wall for a longer period. The latter effect was not observed during the height of the infection, but only during the later stages of the recovery process; therefore, another factor — damage to the cells of the intestinal mucosa — must also play a role.

It appears that early in coccidial infection (near the first day) slight damage to the intestinal cells occurs as the sporozoites attack the cells. This damage and the attendant slight inflammation appear to enhance absorption. Perhaps cells in this condition present a greater surface with greater permeability for small ions such as zinc. Increased cell permeability to certain substances as a result of inflammation due to infection has been discussed by Boyd (8).

As the coccidial attack progresses, damage to cells becomes more severe and intestinal motility decreases (7), but absorption is also decreased as a result of cell destruction; hence the severely curtailed absorption observed near the sixth day post-inoculation. Later, as healing occurs, motility returns to the tract and cellular repair permits absorption rates to return toward the rates prevailing in uninfected birds. During the latter stages of the healing process, approximately 3 to 4 weeks post-inoculation, another period of increased absorption occurs, possibly due to increased permeability of the cells of the intestinal mucosa since intestinal passage rates of infected birds are not different from those of noninfected birds at this time (7).

Most of the inconsistencies in the data from trial to trial appear to be explainable on the basis of the effects of varying amounts of cellular damage upon absorption. Slight damage and inflammation

such as noted at some stages of the infection in the second trial and at the lower levels of inoculation in the other trials appear to increase absorption rates, whereas more severe cellular damage occurring at other stages of infection tends to greatly decrease absorption rates.

The data obtained in these trials tend to confirm the inference drawn from the papers by Stafseth (1) and Erasmus et al. (2) that there is absorptive impairment in birds suffering coccidial infection. This occurs during the first 2 weeks of an attack by *E. necatrix*. Later, absorption rates are increased for a period, thus accounting for the increased growth rates reported by Edgar (3), McLoughlin et al. (4) and Panda and Combs (5) in birds recovering from coccidiosis.

It appears that the growth effects observed during a coccidial infection cycle can be explained largely by consideration of the effects of the disease upon nutrient absorption and intestinal motility. Increased gland weight (5), coccidial toxins (9),[3] hemorrhage and other factors may also have some minor effect upon the growth rate of chicks having coccidiosis.

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# Glucose Oxidation and Glycogen Metabolism in Fluoride-fed Rats [1,2]

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*ABSTRACT* Some of the metabolic fates of glucose were investigated in control and fluoride-fed animals. There was no significant difference in the ability of animals ingesting 450 ppm F to oxidize glucose to carbon dioxide. No relationship was observed between fluoride ingestion and the percentage liver glycogen observed, and blood glucose levels were not altered by fluoride ingestion. Radioactive glucose was incorporated into liver glycogen of both intact control animals and into liver slices from control animals at a faster rate and reached a higher level than was observed in the fluoride-fed rats. Glycogenolysis was greater in liver slices prepared from control animals. The extent of glycogenolysis was independent of the pre-incubation glycogen level which was not altered by the dietary treatment. The addition of fluoride to liver slices from control animals had no effect on the degree of glycogenolysis, and it was concluded that fluoride acts to depress the turnover of liver glycogen, in an indirect manner rather than by direct enzyme inhibition.

Although inorganic fluorides have long been recognized as toxic agents, and numerous studies of the chronic toxicity resulting from their continual ingestion have been reported (1), little is known about the specific metabolic lesions involved. Suttie and Phillips (2) and others (3–5) have demonstrated that there are definite impairments in lipid metabolism, although these effects would not appear to be sufficient to account for the entire toxic nature of inorganic fluorides.

Fluoride ion is known to inhibit a number of enzymes in carbohydrate metabolism (6) and Carlson and Suttie (7) have demonstrated changes in the level of liver glucose-6-phosphate dehydrogenase by fluoride ingestion. In an attempt to identify other systems which may be affected by fluoride, the metabolism of various radioactive compounds by fluoride-fed rats has been investigated. The present studies were designed to investigate the influence of fluoride ingestion on the metabolism of $^{14}C$-labeled glucose by young rats.

## METHODS

*Animals and diets.* Female weanling rats of the Holtzman strain, housed individually in galvanized iron cages with raised screen bottoms in an air conditioned room, were used in all of the reported studies. The animals received distilled water and were fed the control or the 450 ppm F diet (table 1) ad libitum for 30 to 35 days before being used in any investigation. Analysis (8) showed that the control diet contained 4 to 8 ppm F. The inclusion of 450 ppm F in the diet depressed food intake to the extent that the fluoride-fed animals were 30 to 40 g smaller when used in these experiments. Bone fluoride analysis indicated from 10,000 to 16,000 ppm fluoride in the femur ash of the fluoride-fed animals as compared with a normal 400 to 800 ppm fluoride in the control femurs.

In an attempt to equalize as much as possible the pre-experiment nutritional state of the animals, the rats used in the whole-animal studies were fasted overnight for 12 hours and then allowed to feed ad libitum for the next 3 hours immediately preceding the experiment. Following this 12-hour fast the fluoride-fed

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[2] A preliminary report of this work was presented at the 48th Annual Federation meetings, Atlantic City, April, 1964.
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TABLE 1

*Composition of experimental diets*

| | % of diet |
|---|---|
| Casein | 23.5 |
| Sucrose | 66.0 |
| Salts B [1] | 5.0 |
| Corn oil | 5.0 |
| Vitamin premix [2] | 0.5 |

Dietary supplements

(1) 1.0 g choline·HCl/kg diet in 25% ethanol
(2) 0.125 g α-tocopherol/kg diet in corn oil
(3) 1.0 g sodium fluoride/kg diet to fluoride diets, equivalent to 453 ppm F

[1] Harper, A. E., J. Nutrition, 68: 405, 1959.
[2] The vitamin premix was made up in vitamin-free casein and contained the following amounts of vitamins/kg of premix: 1.2 g thiamine·HCl; 0.6 g riboflavin; 0.5 g pyridoxine·HCl; 2.0 g niacin; 0.8 g menadione; 4 g Ca pantothenate; 20 g inositol; 2 mg vitamin $B_{12}$; 40 mg folic acid; 20 mg biotin, 800,000 IU vitamin A and 200,000 IU vitamin D as gelatin beads.

animals consumed almost as much diet in the refeeding period as did the control animals.

*Collection of carbon dioxide.* Each rat received 2.5 μc of carrier-free glucose-UL-$^{14}$C (12 μc/μmole) intraperitoneally and was placed in an all-glass respiration chamber which permitted periodic carbon dioxide samples to be collected. The 3.5-hour collection period was divided into 7 collection intervals of 20, 20, 20, 30, 30, 45 and 45 minutes duration. The carbon dioxide liberated during each time interval was collected in 10 ml ethylene glycol monomethyl ether:monoethanolamine (2:1 v/v), and aliquots of the total sample were taken for $^{14}CO_2$ determination and for determination of total carbon dioxide concentration by titration.

*Isolation and determination of liver glycogen.* Animals were stunned, exsanguinated, and the livers immediately excised and frozen in dry ice until glycogen isolation (9) could be carried out. The precipitated glycogen was washed with 2 successive 25-ml aliquots of 95% ethanol and finally with 25 ml diethyl ether.

*Preparation and incubation of liver slices.* Rat livers were quickly excised, rinsed in Hastings medium I (10), and maintained in ice cold Hastings medium I until sliced free hand (11) and incubated.

For the $^{14}$C-glucose incorporation studies, 1-g portions of liver slices from rats fasted for 12 hours were added to 4 ml Hastings medium I pH 7.4 in 25-ml Erlenmeyer flasks. Slices from 5 control livers were pooled and mixed as were those from 5 fluoride-fed animals before slices were taken for incubation. The flasks were equilibrated on a shaking water bath with 95% $O_2$-5% $CO_2$ at 37° for 10 minutes, and 1.0 ml of medium containing 50 mg glucose to give a final concentration of 1% glucose, and 0.20 ml glucose-UL-$^{14}$C (2.5 μc/ml) was added. The flasks were sealed with rubber serum stoppers, and the incubation continued for another 90 minutes. The reaction in each flask was stopped with 0.5 ml 40% NaOH added by means of a syringe, the tissue from each flask was removed, washed well with the incubation medium, and the glycogen content determined.

In the glycogenolysis experiments, liver slices were prepared from non-fasted rats and a 1-g portion was used to determine the pre-incubation level of glycogen in the tissue. One gram of slices was also incubated in either 3 ml Hastings medium III (12) pH 7.4 or 3 ml Krebs-Ringer phosphate buffer pH 7.4 (11). After equilibration with 95% $O_2$-5% $CO_2$ at 37°, the flasks were sealed with rubber serum stoppers and incubated for 90 minutes. No glucose was added to any of the incubation media.

*Blood glucose determination.* Blood samples were obtained by heart puncture and glucose was determined by the use of a commercially available glucose oxidase kit.[4]

*Radioactivity determinations.* All radioactivity measurements were made in a Packard Tri-Carb liquid scintillation spectrometer, using standard counting techniques. The $^{14}$C activity in expired carbon dioxide was determined by adding a 3-ml aliquot from each of the collected samples to 16 ml toluene:ethylene glycol monomethyl ether (10:6 v/v) containing 5.5 g/liter 2,5-diphenyloxazole (PPO). The radioactivity content of the liver glycogen samples was measured by adding 40 mg of each sample directly to 15 ml of

[4] Glucostat, Worthington Biochemical Corporation, Freehold, New Jersey.
[5] Zebrowski, E. J. 1965 Effects of dietary fluoride on some aspects of carbohydrate and lipid metabolism in the rat. Ph. D. Thesis, University of Wisconsin, Madison.

TABLE 2

*In vivo oxidation of glucose-UL-$^{14}C$*

| Group | Total $CO_2$ | Respiratory rate | % of dose as $^{14}CO_2$ | Specific activity |
|---|---|---|---|---|
| | *mmoles* | *μmoles $CO_2$/ $cm^2$/hr* | | *dpm/mmole $CO_2$* |
| Control (6) | 49.4 ± 3.5 [1] | 41.1 ± 2.6 | 46.4 ± 2.7 | 17300 ± 1240** |
| Fluoride (6) | 36.5 ± 3.4 | 35.7 ± 0.6 | 45.5 ± 3.1 | 22700 ± 1140 |

[1] Values indicated represent the mean ± SE for the number of animals indicated in parentheses.
** $P < 0.01$.

diotol phosphor system (13). The validity of the procedure was determined by comparing it with samples combusted prior to counting.[5]

## RESULTS

The results of the in vivo oxidation of glucose-UL-$^{14}C$ by control and fluoride-fed rats during the 3.5 hours following the intraperitoneal administration of a carrier-free dose (2.5 μc) of the substrate to these animals are shown in table 2. It was found that the control and fluoride-fed animals excreted the same percentage of the administered dose as $^{14}CO_2$. When the respiratory rate was calculated as μmoles $CO_2/cm^2$ surface area/hour, there was no significant difference between the fluoride-fed and control rats. These data indicated that there was no significant difference in the ability of the fluoride-fed animals to oxidize glucose to carbon dioxide. They suggested that the higher specific activity of the carbon dioxide expired by the fluoride-fed rats was the result of a lower expiration of carbon dioxide by the smaller fluoride-fed rats and not of an increased utilization of glucose for carbon dioxide production. No relationship was observed between fluoride ingestion and liver glycogen content, and no significant differences were found in the amount of $^{14}C$ glucose incorporated into liver glycogen at 3.5 hours post-injection.

To clarify and extend the glycogen incorporation data, a time study was carried out to follow the rate of incorporation of the glucose-UL-$^{14}C$ into liver glycogen. Ten fluoride-fed rats and ten control rats, maintained and treated as described, were each given 1.25 μc of glucose-UL-$^{14}C$ intraperitoneally and the animals were killed at intervals of 1, 3, and 5 hours post-injection. Blood glucose concentration was determined in blood obtained by heart puncture immediately before each animal received the labeled glucose and also in blood collected when the animal was killed.

The pattern of glucose-UL-$^{14}C$ incorporation observed is illustrated in figure 1. These data suggested that the liver glycogen in the control animals may have been in a more dynamic state. Radioactivity was incorporated at a faster rate and reached a higher level than noted in the fluoride-fed rats. Once the peak values were reached, the activity declined at a faster rate in the control animals. Since only 3 time periods were taken, the time of peak incorporation could be only approximated. Although these data show a relative retardation or inhibition of glucose-UL-$^{14}C$ incorporation into liver glycogen by the fluoride-fed rats, they do not, however, exclude the possibility that the retardation may be due to an inhibition of

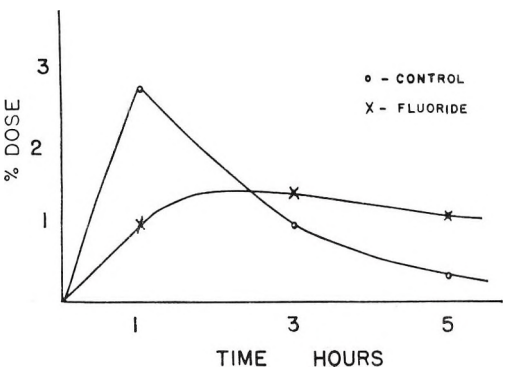


Fig. 1 Incorporation of intraperitoneally administered glucose-UL-$^{14}C$ into liver glycogen isolated from control and fluoride-fed rats at different time intervals. Each point represents the mean of 3 or 4 rats. The values differ significantly at the 1 hour ($P < 0.01$) and 5 hour ($P < 0.05$) periods.

TABLE 3

*Incorporation of glucose-UL-$^{14}C$ into glycogen by liver slices from control and fluoride-fed rats*

| Time | Group | Glycogen | Specific activity | Total dpm incorporated |
|---|---|---|---|---|
| *hours* | | *%* | *dpm/100 mg glycogen* | |
| 1 | Control | 4.0 ± 0.3 [1] | 1815 ± 205 | 700 ± 107* |
| | Fluoride | 3.6 ± 0.3 | 1340 ± 60 | 475 ± 32 |
| 3 | Control | 4.2 ± 0.1 | 1290 ± 135 | 505 ± 41 |
| | Fluoride | 4.0 ± 0.2 | 1170 ± 105 | 445 ± 55 |
| 5 | Control | 4.5 ± 0.3 | 1360 ± 170 | 600 ± 91 |
| | Fluoride | 4.2 ± 0.1 | 1235 ± 30 | 485 ± 12 |

[1] Values represent the mean ± SE for triplicate samples incubated at each time period.
* $P < 0.05$.

absorptive processes. Liver glycogen was found to be in the range of from 4 to 7% of liver weight and there was no significant difference between the amount of liver glycogen in control or fluoride-fed rats at any of the time periods. No differences in blood glucose levels were noted between the control and fluoride-fed rats. A range of from 80 to 110 mg/100 ml blood was observed.

The interference of glycogen metabolism was investigated further by measuring the in vitro incorporation of glucose-UL-$^{14}C$ into glycogen by liver slices (table 3). The specific activity of the glycogen in slices from control rats was significantly greater after 1 hour of incubation, but no differences were observed after 3 and 5 hours. These data are consistent with the incorporation pattern in the whole-animal studies and again suggest that the liver glycogen of the control animals is in a more dynamic state.

The influence of fluoride ingestion on glycogen metabolism was also investigated by determining the relative rates of glycogenolysis in liver slices from these animals.

Three grams of slices were prepared from each liver of non-fasted control and fluoride-fed animals and treated as described. The glycogen content of the incubated slices was determined after 90 minutes' incubation and compared with the pre-incubation glycogen level of the same tissue sample. The results from 2 separate experiments are recorded in table 4. In each case, and in the 2 different media, the liver slices from the control animals degraded about 20% more glycogen than did the liver slices from the fluoride-fed rats. The amount of degradation was found to be independent of the pre-incubation glycogen level of the liver slices which was not significantly altered by dietary treatment. These data offered further evidence of an altered glycogen metabolism in fluoride-fed rats. No indication was given, however, whether the observed impairment was due to a reduced enzyme concentration in the tissue or to a direct inhibition of the enzymes by tissue fluoride.

Although it appeared doubtful that there would be sufficient fluoride in the soft tissues of fluoride-fed rats to directly inhibit glycogenolysis, the effect of fluoride on this system was studied by adding varying amounts of fluoride to slices prepared from rats fed the low fluoride control diet. The results of this study are shown in table 5. No significant differences in the amount of glycogen degrada-

TABLE 4

*In vitro degradation of endogenous glycogen in liver slices from control and fluoride-fed rats* [1]

| Exp. | Group | % Glycogen degraded in 90 min | |
|---|---|---|---|
| | | Krebs-phosphate buffer | Hastings medium III |
| 1 | Control (5) [2] | 67.9** | — |
| | Fluoride (6) | 48.1 | — |
| 2 | Control (5) | 65.3* | 61.7* |
| | Fluoride (3) | 51.7 | 44.0 |

** $P < 0.01$.
* $P < 0.05$.
[1] Pre-incubation glycogen levels: control, 7.15 ± 0.70 mg/100 mg wet weight tissue; Fluoride, 7.40 ± 0.50 mg/100 mg wet weight tissue.
[2] Values reported are the means for the number of animals indicated in parentheses.

TABLE 5

*Effect of sodium fluoride in vitro on glycogen degradation by rat liver slices*

| Group | Fluoride added | | % Glycogen degraded in 90 min |
|---|---|---|---|
| | NaF | $F^-$ | |
| | *M* | *ppm* | |
| Control (8) [1] | 0 | 0 | 61.6 |
| 1 (4) | $1 \times 10^{-4}$ | 1.9 | 60.0 |
| 2 (4) | $5 \times 10^{-4}$ | 9.5 | 58.0 |
| 3 (4) | $1 \times 10^{-3}$ | 19 | 61.0 |
| 4 (4) | $2.5 \times 10^{-3}$ | 47.5 | 55.5 |
| 5 (4) | $5 \times 10^{-3}$ | 95 | 52.5 |
| 6 (4) | $1 \times 10^{-2}$ | 190 | 56.3 |

[1] Values reported are the means for the number of animals indicated in parentheses.

tion were found between the control samples and those to which fluoride had been added. The control samples produced from 54% to 73% degradation of the endogenous glycogen, a range which encompassed that of all fluoride levels used. These data suggest that if fluoride does act to depress the turnover of liver glycogen, then it must act in some manner other than by direct enzyme inhibition.

## DISCUSSION

These studies have demonstrated that rats consuming as much as 450 ppm F in the diet are able to catabolize glucose to $CO_2$ at a normal rate, and to maintain a normal level of liver glycogen. However, an inability of the fluoride-fed rat to metabolize glycogen in a normal manner has been indicated. The demonstration that liver slices from control animals degraded about 20% more endogenous glycogen than did the liver slices from fluoride-fed rats indicates that the observed alterations in glycogen metabolism in intact rats were due to some effect exerted at the liver enzyme level. Two different explanations appeared to be possible. Either the ingested fluoride acted directly to inhibit one or more enzymes involved in glycogen metabolism, or else the absolute level of one or more of the adaptive hepatic enzymes (14, 15) which can be regulated by the nutritional state of the animal is lower in the fluoride-fed rats. As levels of fluoride greatly in excess of those observed for soft tissues had no effect when added to the medium, it was concluded that the effect of fluoride on glycogenolysis was an indirect one.

It appears that fluoride may act indirectly on glycogen metabolism by altering the food consumption and the eating pattern of the fluoride-fed rat, thus affecting liver enzyme concentrations. In preliminary studies, Suttie [6] has observed that fluoride-fed rats not only consume less diet, but eat more often during the day which results in a "nibbling" rather than a normal "meal" type of intake. Carlson and Suttie (7) have demonstrated that the level of glucose-6-phosphate dehydrogenase is decreased in fluoride-fed rats, and that this decrease is mediated through the effect of fluoride on the pattern of food consumption. It has also been demonstrated that several of the enzymes concerned with glycogen metabolism in the liver (15) can be varied by regulating food intake. It is likely that the level of the adaptive hepatic enzymes regulating glycogen turnover might therefore be reduced in the fluoride-fed animals, and such a reduced enzyme level could readily account for the impaired glycogen turnover rate observed in these studies.

These observations point out a defect in carbohydrate metabolism and add to the list of metabolic changes observed in animals fed toxic amounts of fluoride. The evidence also indicates that this defect is probably secondary to a more direct effect of fluoride on some other area of metabolism.

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# Chronic Skin Homograft Rejection in Vitamin $B_6$-deficient Mice [1]

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*ABSTRACT* A study was made of the effects on skin homograft survival times in strain C57 mice given a diet deficient in pyridoxine (vitamin $B_6$). The pyridoxine-deficient mice showed a prolonged survival time for the homografts and exhibited a chronic homograft rejection pattern. The cellular details of this chronic rejection are presented in a series of photomicrographs which illustrate how the chronic type differs from the usual acute homograft reaction.

Previous reports (1–3) indicated that pyridoxine (vitamin $B_6$) deficiency led to a prolonged survival of skin homografts in adult rats. Significant increases in survival times of skin homografts (4) and ovarian homografts (5) have also been obtained in pyridoxine-deficient mice. Humphries et al. (6) have reported that chronically vitamin $B_6$ deficient dogs tolerated skin homografts longer than did normal control animals. On the other hand, Fisher (7) was unable to discover an influence on skin homograft survival times in pyridoxine-deficient humans.

The present paper is concerned with a further study of the effects associated with pyridoxine deficiency on skin homografts and homograft survival times in C57 mice.

## MATERIALS AND METHODS

The JAX mice [3] used in this experiment were of strains C57BL/6J and 129/J and were bred and maintained at the Franklin and Marshall Biology Department. These inbred-derived mice were fed water and commercial mouse breeder chow [4] ad libitum.

To produce the deficiency, recipient C57 mice were fed a commercial pyridoxine-deficient diet [5] and treated with approximately 0.05 mg of the pyridoxine antagonist, deoxypyridoxine hydrochloride ($DB_6$)/day/mouse, as an additive to the drinking water (4). The concentration in water depended upon the determined daily intake. Control C57 recipients were also fed the deficient diet. Instead of $DB_6$, the control mice received 0.05 mg of pyridoxine hydrochloride/day/mouse in their drinking water. The mice were housed in individual cages.

As a preventive against possible production of vitamin $B_6$ in the alimentary tract by the intestinal flora, an antibiotic [6] was added to the drinking water of all experimental animals at a dosage of 0.05 mg/day/mouse. The mice were skin grafted after 9 days of the above treatment. After 14 days, the pyridoxine-deficient diet was discontinued and all mice were returned to the mouse breeder chow.

Early in the experiment (day 14) the additives to the drinking water (vitamin $B_6$ and the antibiotic for control mice, and $DB_6$ and the antibiotic for deficient mice)

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[3] Obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine.

[4] Purina Mouse Breeder Chow, Ralston Purina Company, St. Louis.

[5] The vitamin $B_6$-deficient diet contained: (in %) vitamin-free test casein, 18; sucrose, 67; vegetable oil, 10; salt mixture no. 2 USP, 4; and vitamin fortification mixture (pyridoxine-free), 1. The vitamin fortification mixture contained/kg of diet: (in grams) vitamin A conc (200,000 U/g), 0.0990; vitamin D conc (400,000 U/g), 0.0055; α-tocopherol, 0.1102; ascorbic acid, 0.9920; inositol, 0.1102; choline chloride, 1.6534; menadione, 0.0496; *p*-aminobenzoic acid, 0.1102; niacin, 0.0992; riboflavin, 0.0220; thiamine·HCl, 0.0220; Ca pantothenate, 0.0661; and (in milligrams) biotin, 0.441; folic acid, 1.984; and vitamin $B_{12}$, 0.030. Salt mixture no. 2 USP contained: (in %) Ca lactate, 32.69; Ca biphosphate (monobasic), 13.58; ferric citrate, 2.96; magnesium sulfate, 13.70; potassium phosphate (dibasic), 23.99; sodium biphosphate (monobasic), 8.73; and sodium chloride, 4.34.

[6] Humatin, Parke, Davis and Company, Detroit.

were also discontinued. At that time, some control animals and many deficient animals developed diarrhea with resulting fecal impactions. The diarrhea could sometimes be relieved if the antibiotic was again added to the drinking water of affected mice. Later in the experiment an attempt was made to reduce the incidence of diarrhea by continuing the antibiotic treatment without interruption until the grafts were rejected.

Full thickness donor skin grafts, approximately 1 $cm^2$, were removed from the abdomens of untreated strain 129 female mice and placed on prepared fitted graft beds on the dorsolateral thorax areas of host C57 male and female mice. The techniques used in preparing the donor grafts and recipient graft beds, and for casting of mice, are described by Billingham (8). Two modifications of the above techniques were utilized in this experiment:

1) In later phases of the experiment the grafts were sutured to their graft beds at each corner, using number 60 black cotton thread, to eliminate the number of "no takes" (failure of graft to make establishment with its bed) and "partial takes" (partial establishment of graft) due to movement of the graft during or after casting. The sutures were removed on the seventh postoperative day. No effect on duration of graft survival times was noted by this change in technique.

2) After removal of the original plaster cast, the grafts were again covered with a piece of vaseline gauze and a lightweight paper cast fashioned from a 9-cm long piece of 1.3-cm laboratory tape.[7] The vaseline gauze and paper casts were changed as needed up to the day of graft rejection.

Grafts of control and deficient mice were examined with a stereoscopic microscope on the seventh and ninth postoperative days. Thereafter, control grafts were observed daily until they were rejected. Grafts from the vitamin $B_6$-deficient mice were observed on alternate days until rejected. When an animal died or rejected a graft during the course of the experiment, its graft site was removed and saved for possible histological study. In addition, to obtain a histological sequence of the deficient homograft rejection phenomenon, deficient mice were killed at intervals and their graft sites removed, fixed in 10% formalin, and sectioned at 5 μ. The routine hematoxylin-eosin staining procedure was used on all sections.

A graft was considered as rejected on the day of complete sloughing. Where interpretations from gross observations were difficult, graft sites were removed for histological study. The median survival time, as computed from the nomograph method of Litchfield (9) was used to measure the intensity of the reaction which homografts elicited from their hosts.

Approximately 0.3 ml of blood was removed from the tails of all deficient and control mice on the day dietary treatment was initiated, the day of grafting, the ninth postoperative day, and at 3- to 4-day intervals thereafter, to determine the changes in blood proteins before, during, and at the termination of the homotransplantation rejection phenomenon. The results of this study will be presented in a subsequent publication.

## RESULTS

Both control and deficient mice experienced a change in weight after 9 days of dietary treatment (table 1). By the end of the entire 14-day treatment period, most of the deficient mice had developed mild dermatitis of the tail and paws, as well as the other characteristics of "acute pyridoxine deficiency" (10). In addition, the deficient mice proved to be more susceptible to overdoses of anesthesia than were the control animals.

Prolongation of the antibiotic treatment was moderately effective in reducing the number of diarrheal attacks among the deficient animals. This prolongated treatment, in addition, introduced a high incidence of abdominal distension in the deficient group (table 2). This distension, which was caused by an accumulation of peritoneal fluid, was first noticed on about the fifteenth day of dietary treatment and increased in size for another two or three days. During this period, in some instances, the weights of afflicted mice increased 3, 4, or even 6 g. The disten-

[7] Time Labels, Professional Tape Company, Inc., Riverside, Illinois.

TABLE 1

*Age and weight data for recipient C57 mice* [1]

| Experimental group | Age [2] | Body weight | | |
|---|---|---|---|---|
| | | Initial | Final [3] | Net wt change |
| | *weeks* | *g* | *g* | *%* |
| Control males | 6 | 17.3 ± 0.5 [4] | 20.6 ± 0.3 | + 19.1 |
| Control females | 6 | 15.7 ± 0.6 | 18.1 ± 0.6 | + 15.3 |
| Pyridoxine-deficient males | 7 | 20.8 ± 0.5 | 19.5 ± 0.4 | − 6.2 |
| Pyridoxine-deficient females | 7 | 17.1 ± 0.6 | 16.2 ± 0.7 | − 5.3 |

[1] Data refer only to mice listed in table 3.
[2] Approximate age on day dietary treatment was begun.
[3] Weight on day mice were grafted.
[4] Mean ± SE of mean.

TABLE 2

*Results of prolongation of treatment time with antibiotic* [1]

| Experimental group | Total no. of mice | Duration of treatment | No. of mice afflicted | | |
|---|---|---|---|---|---|
| | | | Fecal impaction | Died | Abdominal distension |
| Control | 17 | 14 days | 3 | – | 1 |
| Control | 10 | to rejection | 2 | – | — |
| Pyridoxine-deficient | 18 | 14 days | 8 | 3 | — |
| Pyridoxine-deficient | 25 | to rejection | 7 | 2 | 13 |

[1] Humatin, Parke, Davis and Company, Detroit.

sion persisted for no more than 3 or 4 days and usually disappeared as rapidly as it had appeared. No deaths were attributable to this abdominal distension and its presence did not appear to have any effect on graft rejection times.

The grafts which were sutured to their graft beds remained in place and could all be considered as initial "takes." The paper casts were extremely well-tolerated by all experimental mice. Most mice gnawed at their original plaster casts, even with the coating of 1% picric acid recommended by Billingham (8) to discourage this habit. With the uncoated paper casts this difficulty was not encountered.

*Microscopic studies of the rejection process.* Control grafts, where observed grossly, appeared to follow the usual acute homograft reaction. This reaction, once begun, proceeded rapidly to completion in 3 to 5 days. The vaseline gauze used to cover the grafts prevented drying of the graft sites. This facilitated graft appraisal since, when all connections with the graft bed were severed, the entire necrotic graft could usually be lifted away to reveal the new host epidermis (fig. 1).

The grafts of deficient mice were much more difficult to appraise and appeared to undergo a more chronic type of rejection. The grafts were not rejected uniformly. Instead, they first became eroded around their edges and gradually decreased in size, with accompanying contracture of their graft beds. Small portions of these grafts, later found to be collagen pads, sometimes remained weakly attached to a graft site for as long as 5 days. Other grafts from vitamin $B_6$-deficient animals underwent a slow transformation into shiny bald scars.

The graft rejection process in deficient animals could be best interpreted histologically. On about the thirteenth day after grafting, a round cell infiltration began to appear beneath the graft dermis (fig. 2). This infiltration became very intense and soon involved the entire graft, with resulting hyalinization of the graft dermis and epidermis, vacuolization of the epidermis and its appendages, and rupture of graft blood vessels (figs. 3 and 4). By the twentieth day the dermis and epidermis at the periphery of the graft were densely infiltrated with invading cells and highly disorganized. Concomi-

TABLE 3

*Median survival times of female 129 mouse skin grafted orthotopically to male and female C57 mice*

| Experimental group | No. of mice | Median survival time | Survival time (range) |
|---|---|---|---|
| | | *days* | *days* |
| Control males | 12 | 13.2 ± 1.97 [1] | 12–17 |
| Control females | 10 | 12.4 ± 1.07 | 12–17 |
| Pyridoxine-deficient males | 9 | 22.7 ± 5.10 | 18–35 |
| Pyridoxine-deficient females | 8 | 22.9 ± 1.26 | 21–25 |

[1] SD.

tantly with the sloughing of these disorganized areas, host migratory epithelium began to grow inward from the edges of the graft bed. Sometimes it was noted that the rapidly proliferating epidermis overgrew parts of what appeared to be hyalinized graft dermis (figs. 5 and 6). Areas of apparently similar overgrowth of graft dermis were also observed in a control graft site (fig. 1). By the twenty-third day the migratory epidermis had usually covered the entire graft site. After completion of epidermization by the host, the cellular infiltration usually subsided and the new epidermis began to develop appendages (fig. 7). At this time newly formed subepidermal blood vessels could usually be seen in histological sections. Histologically determined rejection times were recorded as the day of complete sloughing of all donor tissue, with the possible exception of small areas of overgrown hyalinized graft dermis. Persistent collagen pads were considered rejected when completely undermined by host epidermis.

The median survival times of grafts for the mice used in this experiment are tabulated in table 3. No significant difference was found between the median survival time of male and female mice within each experimental group ($P > 0.3$ and $P > 0.9$, for control and deficient groups, respectively). For both sexes, however, the difference between the median survival times of control and deficient mice was highly significant ($P < 0.001$).

One deficient male mouse had a graft which remained in excellent condition for 32 days, during which time the graft produced a full growth of new hair. The rejection of this graft, which began on the thirty-second day and lasted only 3 days, followed the acute homograft rejection pattern.

## DISCUSSION

The exact mode of action by which pyridoxine deficiency enhances the survival times of skin homografts has yet to be elucidated. Stoerk (10) suggested that it is the formation of antibody protein which is impaired by the vitamin $B_6$ deficiency. The profound lymphatic atrophy accompanying acute pyridoxine deficiency probably exerts an important supplementary role by drastically limiting the number of lymphocytes available for transport of antibody to graft sites. The concept offered by Fisher et al. (11) that blockade of the reticuloendothelial system prolongs graft survival remains to be proved.

That the control and deficient mice rejection patterns were dissimilar was obvious from gross examination. The control mice rejected their grafts by the usual acute homograft rejection process, whereas the vitamin $B_6$-deficient mice exhibited what can best be described as a chronic homograft rejection process. Before the histological sections were studied, graft survival times for deficient mice were in some instances exaggerated due to the persistence of collagen pads which sometimes remained weakly attached to their graft beds for as long as 5 days. Upon histological examination it was found that these pads were undermined by host epidermis and hence could be considered to be rejected. Hildemann and Walford (12) mentioned similar collagen pads in their description of "rapid chronic homograft rejections" by the Syrian hamster. From both a gross and microscopic viewpoint, this

phenomenon in the hamster parallels the chronic rejection of homografts by the vitamin $B_6$-deficient mice in this experiment.

The details of cellular infiltration during acute homograft rejection have been described by Steinmuller (13), Chutna (14) and others. These investigators all report the development of a marked round cell infiltration during the course of rejection, with a subsequent infiltration of granulocytes. The histological observations made in this experiment correspond closely with the above sequence of events, except that in the cases of pyridoxine-deficient mice the onset of cellular infiltration was delayed and the total rejection time was greatly prolonged. Titus and Shorter (15), on the other hand, have presented the consistent observation that neutrophilic polymorphonuclear leukocytes constitute the predominant component of the cellular reaction at the homograft rejection site. These authors mention that infection cannot be used to explain their results and that they are otherwise unable to account for this variation of the usually observed sequence of events during homograft destruction.

The rejection pattern for the mouse whose graft persisted for 35 days (table 3) followed more closely an acute homograft rejection process than the chronic-type process characteristic of the other deficient mice. Probably the acute rejection occurred because at 35 days this animal was no longer severely pyridoxine-deficient, and hence responded more like a control animal to the grafted skin.

According to the manufacturer of the antibiotic used in this experiment, it is not appreciably absorbed from the gastrointestinal tract, even following exceptionally high doses. Thus any differences in graft survival times would not be expected to be due to an alteration of the time during which this drug was administered. The diarrhea which developed in many experimental mice was probably caused by an organism which ordinarily is held in balance by the rest of the intestinal flora. Cultures of fecal material from afflicted mice indicated that this organism was *Escherichia coli*, but the exact strain was not ascertained.

The recorded weight gains for control mice (table 1) include a natural gain in weight due to growth, since the 6-week-old mice used were not yet fully grown. The losses of weight for deficient mice should also be considered in this light. They can be viewed as an underestimate of the actual weight losses incurred from the pyridoxine deficiency. Hargis et al. (4) using fully grown C57 mice, weighing about 24 g, reported a weight loss of 17% for mice maintained on a comparable vitamin $B_6$-deficient dietary regimen for 8 or 9 days. Thus, while pyridoxine deficiency may exert a specific effect of the immune responses, the alterations on the overall metabolism of the host, such as manifested in the above weight losses, should not be ignored.

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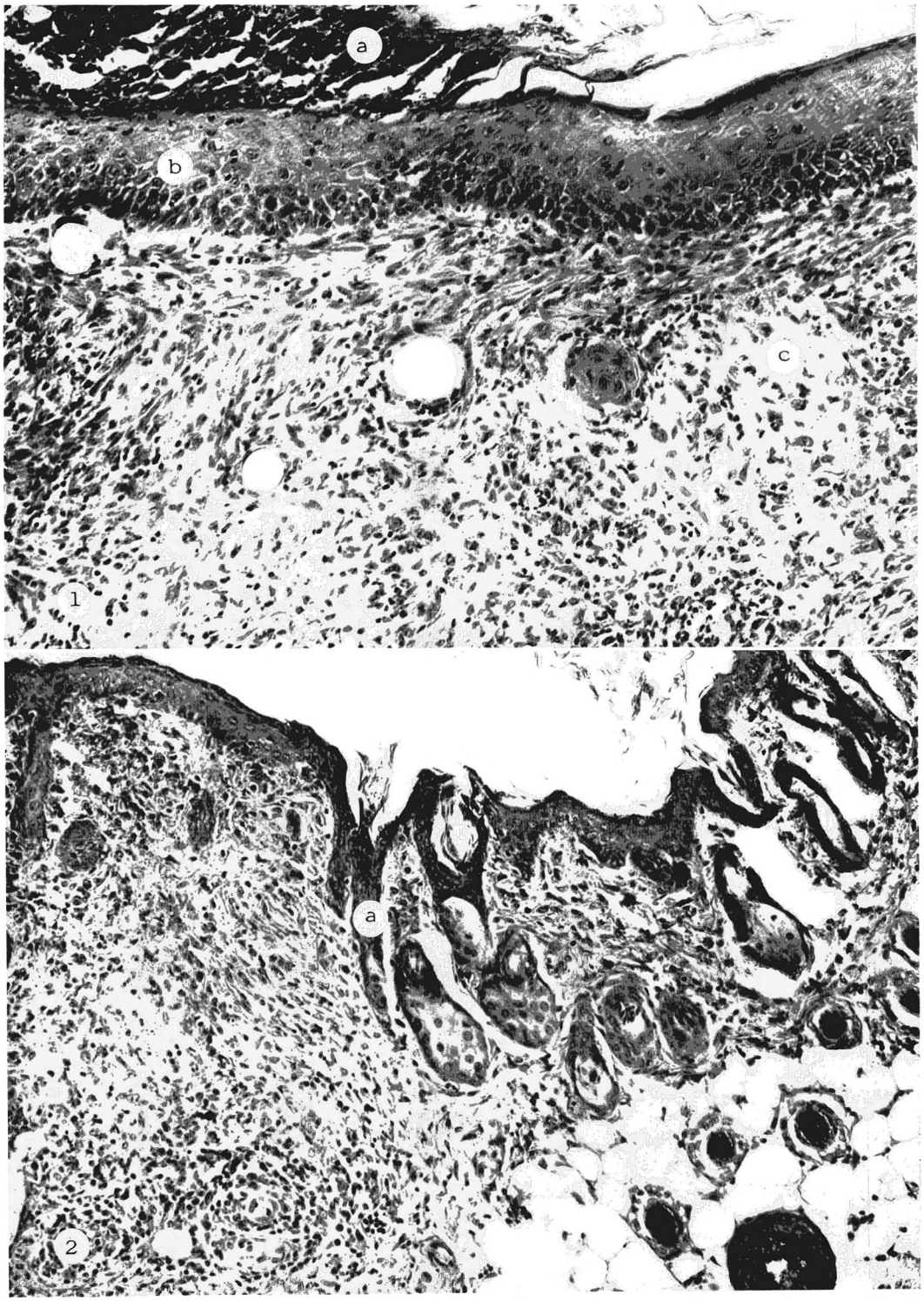
## PLATE 1

### EXPLANATION OF FIGURES

1 Control graft site 12 days after transplantation. Note the disorganized remains of the rejected graft (*a*) above the new host epidermis (*b*). What appear to be persisting patches of hyalinized graft dermis (c) are also visible. ×187.

2 Area of fusion between skin of graft (left) and deficient host (right) 13 days after transplantation. Note host-graft junction (*a*). A round cell infiltration may be observed beneath the graft dermis (lower left). × 187.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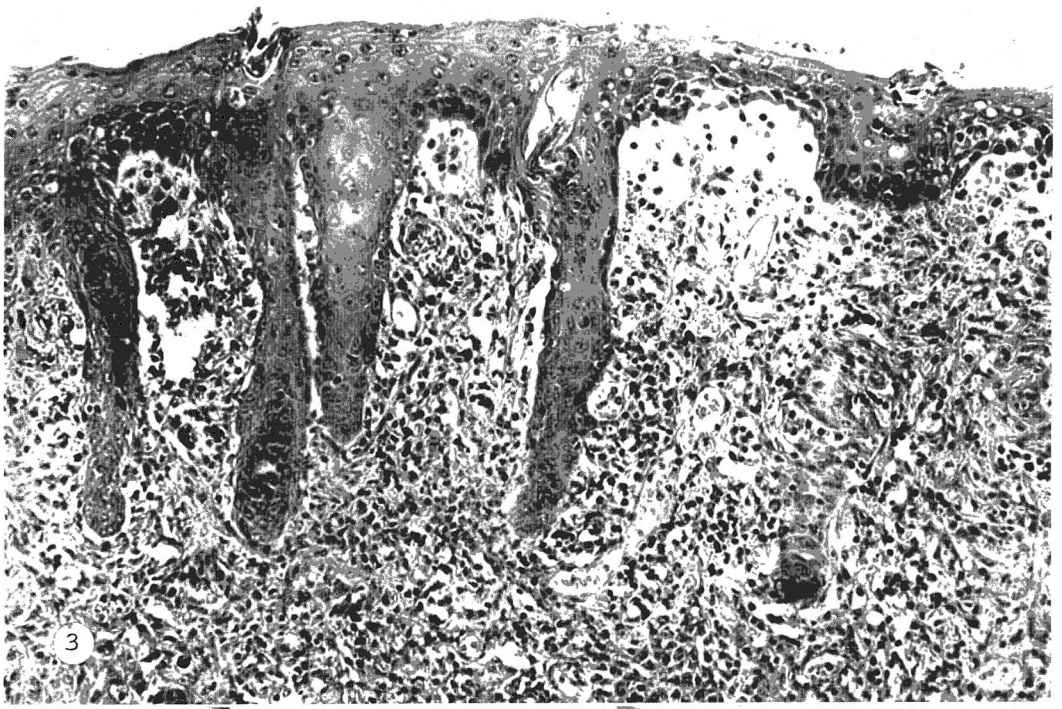
## PLATE 2

### EXPLANATION OF FIGURES

3 Graft of deficient mouse at 16 days showing infiltrating round cells. Note extensive hyalinization and early breakdown of epidermal appendages. × 187.

4 Enlarged area of above epidermis showing round cells infiltrating epidermis and accompanying disorganization. Note vacuolization of prickle layer (*a*). A ruptured blood vessel (*b*) may be seen in dermis. × 663.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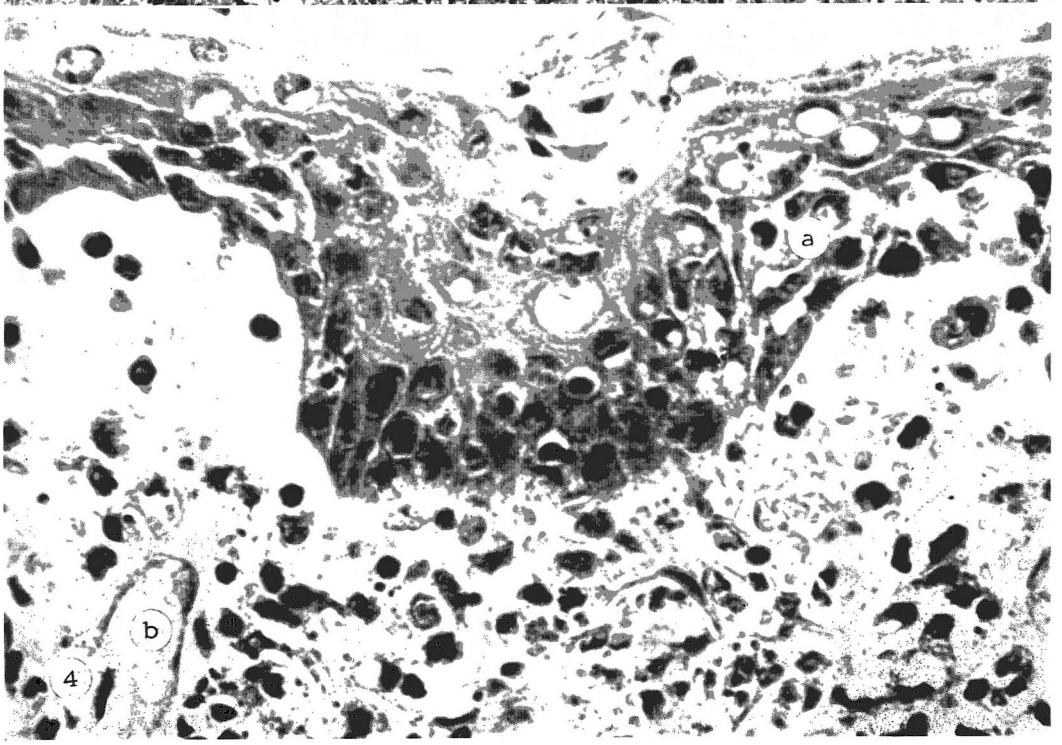
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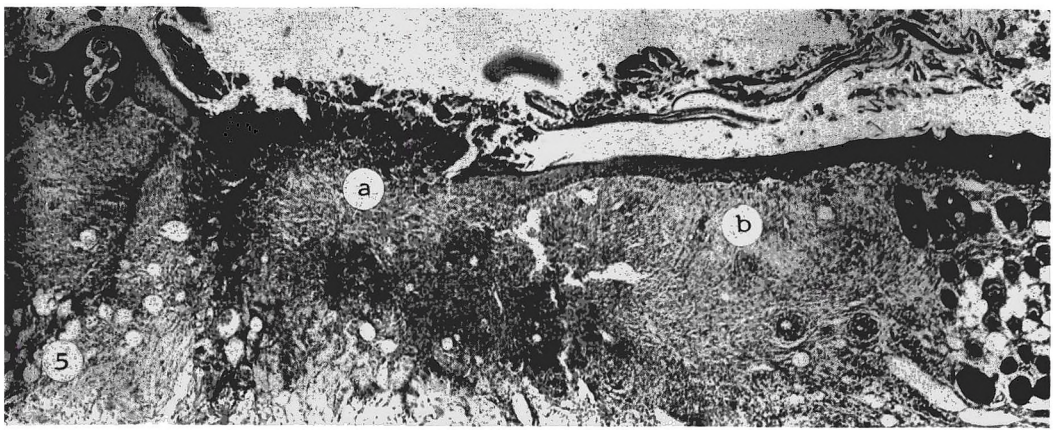
## PLATE 3

EXPLANATION OF FIGURES

5 Graft site of deficient mouse at 23 days illustrating rejection process of graft and subsequent epidermization by host. Note area where this process is not yet completed (*a*) and small patches of persisting hyalinized graft dermis (*b*) which were overgrown by host migratory epithelium. × 51.

6 Enlarged portion of the above migratory epithelium. Note the dense cellularity in area where active rejection is taking place (left). The overgrown portions of graft dermis are again noted (*a*). × 153.

7 Graft site of deficient mouse at 36 days. The graft was rejected on the previous day. Note similarity to control graft site (fig. 1). Also note newly formed blood vessels and epidermal appendages. × 187.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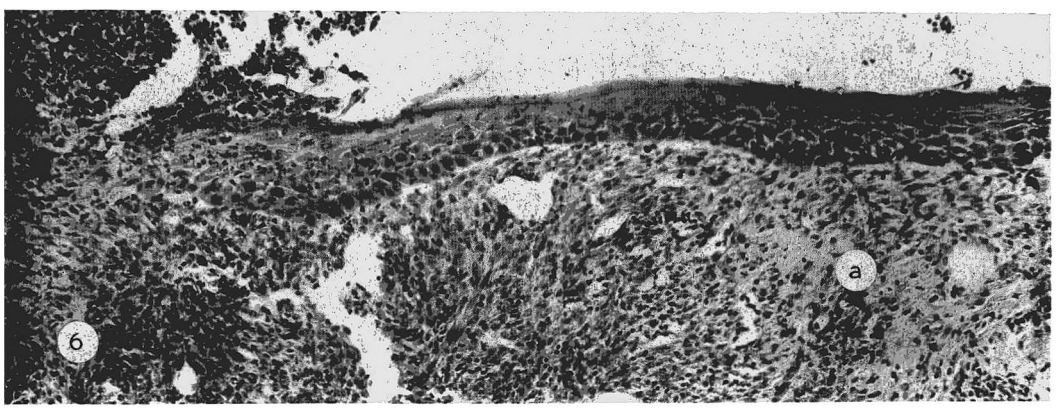
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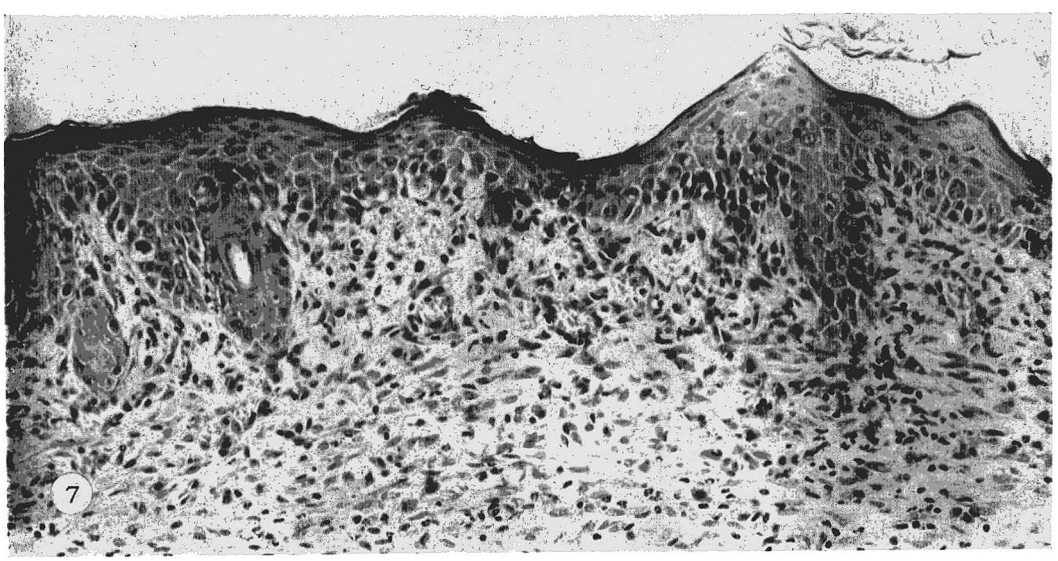
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# Influence of Dietary Calcium, Zinc, and Oil upon the in vitro Uptake of Zinc-65 by Porcine Blood Cells [1]

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*ABSTRACT* Twenty-four weanling barrows averaging 19 kg were allotted to 6 lots and fed at variable levels of either calcium, zinc, or fat for 16 weeks to determine effects on $^{65}Zn$ retention by porcine blood cells in vitro. Five milliliters of freshly drawn whole blood were obtained at 2-week intervals and dosed with 200 mμc $^{65}Zn$. After incubation under $O_2$ atmosphere at 38° for a 2-hour period, the cells were separated from the plasma and their radioactive zinc content was determined. Average percentage cellular $^{65}Zn$ uptake per treatment after packed-cell volumes were adjusted to 40 were: basal, 34; basal + 71 ppm Zn, 26; basal + 0.6% Ca, 55; basal + 71 ppm Zn + 0.6% Ca, 28; basal + 5% oil, 34; basal + 0.6% Ca + 5% oil, 51. Dietary calcium increased ($P < 0.01$), whereas dietary zinc decreased ($P < 0.01$) in vitro uptake $^{65}Zn$ throughout the experiment. Added oil exerted no significant effect on $^{65}Zn$ uptake in vitro. Dietary treatment had no detectable effect on the stable zinc content of these cells. The effect of treatment upon the in vitro uptake of $^{75}Se$, $^{137}Cs$, $^{131}I$, $^{60}Co$, $^{54}Mn$, $^{59}Fe$, $^{115}Cd$, and $^{64}Cu$ by swine blood cells was also investigated with variable results. Four incidences of parakeratosis were observed in the 8 pigs fed the high calcium-low zinc diets.

The mechanism whereby calcium aggravates zinc deficiency in swine (1–3), chickens (4), and rats (5) has not been well established. After thoroughly reviewing the subject of calcium-zinc interrelationship, Forbes (6) and Hoekstra (7) concluded that calcium exhibited its aggravating effect by either interfering with zinc absorption from the gastrointestinal tract, or by interacting with zinc at specific cellular sites within the body.

Hanson (8) prevented and alleviated the parakeratotic syndrome by the addition of 23% soybean oil to swine rations. Phosphorus (9), copper and iron (10),[4] cadmium (11), and source of dietary protein (12) have also been reported to be involved in zinc deficiency.

The present study was conducted to ascertain whether the in vitro uptake of $^{65}Zn$ by porcine blood cells could be altered by dietary calcium, zinc, or corn oil and to compare uptake of various other ions.

## MATERIALS AND METHODS

In each of 2 replicate trials, 12 weanling barrows averaging 19 kg were allotted to the following 6 dietary treatments: basal; basal + 71 ppm zinc; basal + 0.6% calcium; basal + 71 ppm zinc + 0.6% calcium; basal + 5% corn oil; and basal + 5% corn oil + 0.6% calcium. The corn-soybean meal basal (table 1) contained 0.56% calcium, 0.55% phosphorus, and 29 ppm zinc. Supplemental calcium and zinc were supplied as $CaCO_3$ and $ZnSO_4$, respectively. The pigs were treated for external and internal parasites one week before being placed on experiment. To prevent consumption of zinc other than that supplied in the rations, animals were given distilled water ad libitum and galvanized feeders and waterers were painted with 3 coats of liquid plastic. The pigs were kept 2 per concrete pen for 8 weeks and then separated into individual pens for the remainder of the experiment. No bedding was provided and animals were fed their respective diets ad libitum for a total of 16 weeks.

Five milliliters of freshly drawn heparinized whole blood were obtained at 2-week intervals and incubated with 200 mμc $^{65}Zn$

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[2] Present address: Monsanto Company, Agricultural Division, 800 N. Lindbergh Boulevard, St. Louis, Missouri.

[3] Operated by the Tennessee Agricultural Experiment Station for the U. S. Atomic Energy Commission under Contract no. AT-40-1-GEN-242.

[4] Hoefer, J. A., D. E. Ullrey, E. R. Miller, R. W. Luecke and H. D. Ritchie 1958 The effect of feeding zinc, iron and copper to pigs from weanling to market weight. J. Animal Sci., 17: 1186 (abstract).

TABLE 1
*Basal ration*

| | % |
|---|---|
| Corn | 74.50 |
| Soybean meal | 18.00 |
| Alfalfa meal | 2.76 |
| Fish meal | 2.50 |
| $CaHPO_4$ | 1.04 |
| Antibiotic supplement [1] | 0.33 |
| Salt | 0.50 |
| $CaCO_3$ | 0.23 |
| Vitamin premix [2] | 0.08 |
| Trace mineral mix [3] | 0.06 |

[1] Provided 22 mg oxytetracycline and 22 μg cyanocobalamin/kg of feed.

[2] Vitamin premix supplied/kg ration: vitamin A, 1375 IU; vitamin D, 275 IU; and (in milligrams) riboflavin, 2.6; Ca pantothenate, 11.0; niacin, 13.6; pyridoxine, 1.3; choline chloride, 20.7; ethoxyquin (Santoquin, Monsanto Chemical Co., St. Louis), 124.7; and methionine, 499.

[3] Trace mineral mix supplied/kg ration: (in milligrams) cobalt, 0.9; iodine, 0.2; manganese, 49.1; iron, 84.5; and copper, 21.3.

(> 10576 mc/g Zn) contained in 50 microliters of isotonic saline according to the procedures outlined by Wright and Bell (13). Blood samples were incubated at 38° under a 95% oxygen, 5% carbon dioxide atmosphere for 2 hours. After incubation, cells were washed twice with 20 volumes of physiological saline and separated from plasma by refrigerated centrifugation. Gamma counting was carried out in the washing tubes in a well-type scintillation counter. Activity is expressed as percentage radioactive uptake by the cellular fractions of whole blood after packed-cell volumes were adjusted to 40. Blood samples were also obtained at random between the sixth and fourteenth week and incubated with either $^{131}$I, $^{75}$Se, $^{137}$Cs, $^{60}$Co, $^{54}$Mn, $^{59}$Fe, $^{115}$Cd, or $^{64}$Cu.

Blood samples taken at 2-week intervals and feed samples were analyzed for stable zinc by atomic absorption spectrophotometry with slight modification of the method of Fuwa et al. (14). To minimize zinc contamination in these assays, only triple-distilled water was used and all glassware and crucibles were boiled in 6 N HCl for 2 hours and rinsed in triple-distilled water. The colorimetric method of Fiske and Subbarow (15) was used for chemical analysis of feed phosphorus. The calcium content of each feed ingredient was determined by atomic absorption spectrophotometry.[5] Data were subjected to statistical analyses, using procedures outlined by

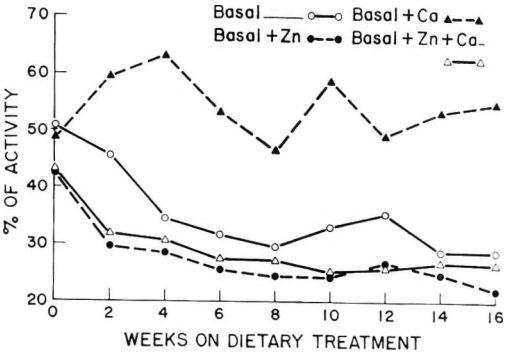


Fig. 1 Influence of Ca and Zn upon in vitro uptake of $^{65}$Zn by porcine blood cell.

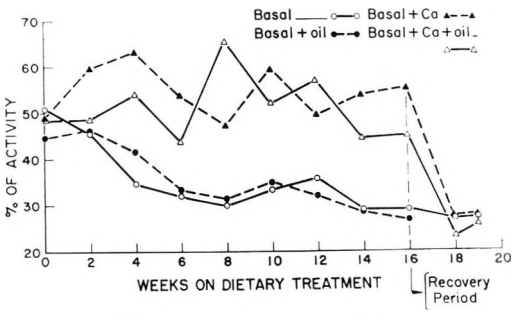


Fig. 2 Influence of Ca and oil upon in vitro uptake of $^{65}$Zn by porcine blood cell.

Snedecor (17). These data were subjected to analysis of variance treatment and analyzed separately as Ca × Zn and Ca × oil 2 × 2 factorial designs.

## RESULTS

The effect of calcium and zinc upon in vitro uptake of $^{65}$Zn by porcine blood cells is presented in figure 1. Dietary calcium increased ($P < 0.01$), whereas zinc decreased ($P < 0.01$) in vitro uptake of $^{65}$Zn throughout the 16-week experiment. Since calcium increased activity ($P < 0.01$) when added to a low zinc ration and had no observable effect on $^{65}$Zn uptake when added to a zinc supplemented ration, a Ca × Zn interaction is obvious (fig. 1). Studies by Wright and Bell (13) have revealed that the in vitro uptake of radioactive minerals by blood cells can be influenced by dietary treatment. They demon-

[5] Perkin-Elmer Manual. Analytical Methods for Atomic Absorption Spectrophotometry. Perkin-Elmer, Norwalk, Connecticut.

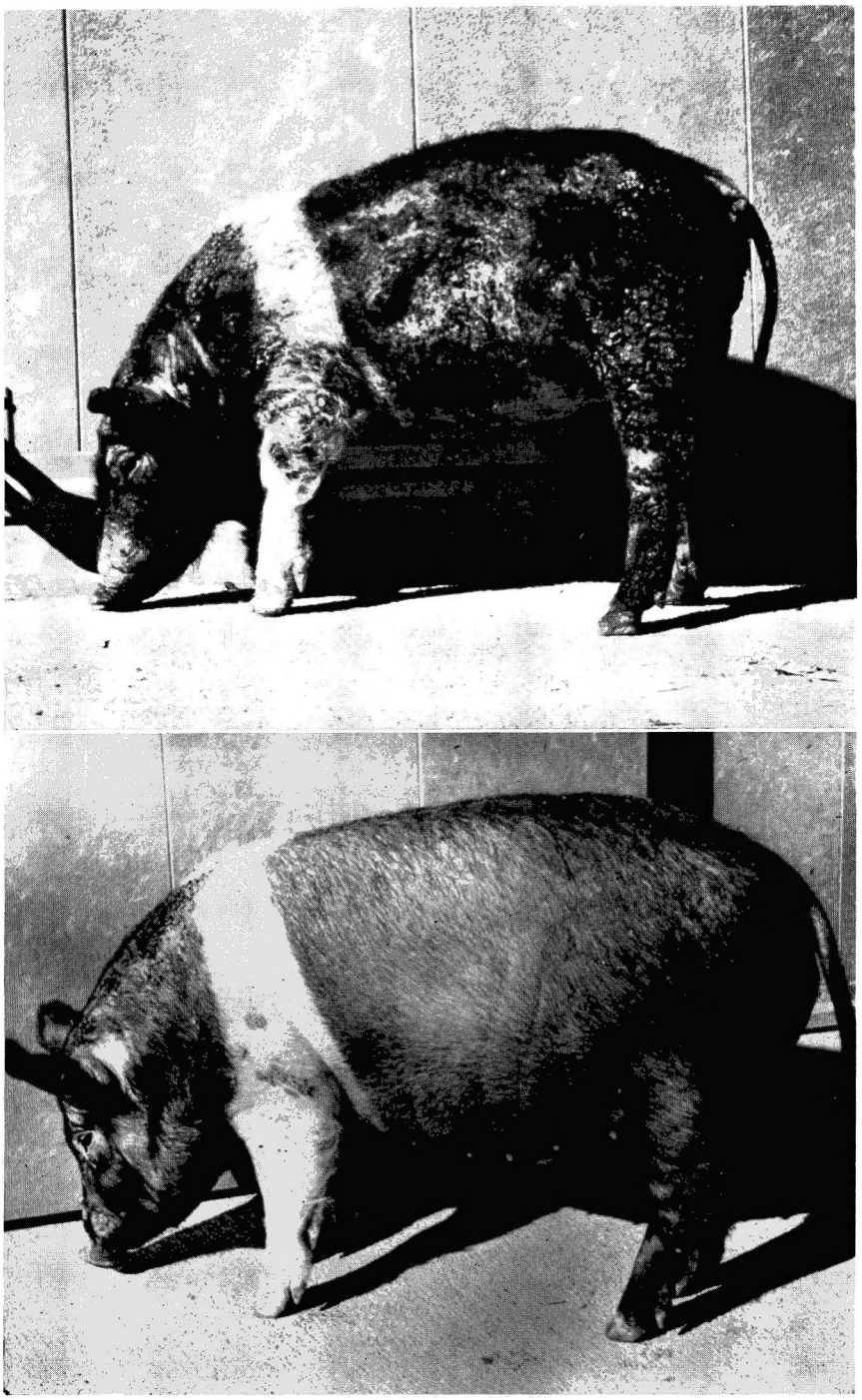

Fig. 3 Therapeutic value of zinc on a pig exhibiting severe parakeratotic lesions. (*upper*) Pig after receiving high Ca-low Zn ration for 16 weeks. (*lower*) Same animal, following 9 weeks of 71 ppm zinc supplementation.

strated $^{75}$Se uptake by ovine blood cells to be inversely proportional to dietary intake of selenium.

Data in figures 1 and 2 show a general decline of $^{65}$Zn uptake by porcine blood cells as the animals increase in age. This is assumed to be an aging effect; however, it could be that all the pigs were borderline zinc-deficient at the start of the experiment.

Data in figure 2 reveal the effect of calcium and oil on blood cellular $^{65}$Zn activity. Dietary oil exerted no significant effect and the calcium plus oil treatment effect is similar to that of supplemental calcium alone. During a 3-week recovery period from weeks 16 to 19, pigs on treatments 3 (basal + Ca), and 6 (basal + oil + Ca) received 71 ppm supplemental zinc. During this period, in vitro $^{65}$Zn uptake by blood cells from these animals quickly decreased to that of the controls.

Photographs of one of the more severely affected parakeratotic pigs after 16 weeks of receiving a zinc-deficient ration and after 9 weeks of recovery following zinc supplementation are shown in figure 3. This pig gained 68 g/day during the first 16 weeks and 1022 g/day during the last 9 weeks. Recovery was very rapid when 71 ppm zinc were added to the diet which is in agreement with observations noted in zinc-deficient calves by Miller et al. (17).

Dietary calcium or zinc had only a minimal effect on the amount of stable zinc in these blood cells (table 2). Packed-cell volumes were adjusted to 40 for calculation of stable zinc in the cellular fraction. Compared with other swine on study, parakeratotic pigs had slightly lower hematocrit readings. Hoekstra et al. (18) reported similar results where the addition of 2% bonemeal did not significantly alter the zinc content of erythrocytes even when parakeratosis was produced in pigs.

Table 3 records the effects of calcium, zinc, and oil upon in vitro uptake of $^{131}$I, $^{75}$Se, $^{137}$Cs, $^{60}$Co, $^{54}$Mn, $^{59}$Fe, $^{115}$Cd, $^{64}$Cu, and $^{65}$Zn. The $^{65}$Zn values are the average of all $^{65}$Zn incubation during the 16-week study. All other radioactivity values were obtained from one duplicate-sample incubation per animal. Calcium increased $^{137}$Cs retention ($P < 0.05$). $^{59}$Fe in vitro uptake was variable among animals; however a significant calcium-zinc interaction ($P < 0.05$) was observed. Calcium and zinc increased $^{59}$Fe uptake when added to the ration separately; however, when both were added simultaneously, they decreased $^{59}$Fe activity. Dietary zinc in the presence of added calcium decreased in vitro $^{64}$Cu activity ($P < 0.05$) of these blood cells. $^{131}$I, $^{75}$Se, $^{60}$Co, $^{54}$Mn, and $^{115}$Cd retention were not significantly affected by dietary treatment. Although variable results were obtained, a general trend was observed for increased in vitro uptake of all ions studied by blood cells from pigs fed elevated calcium-low zinc diets.

Calcium significantly decreased ($P < 0.05$) daily feed consumption and rate of gain of these pigs (table 4). Feed efficiency was not significantly affected by diet. Parakeratosis was observed in 4 of the 8 pigs fed the high calcium-low zinc rations.

## DISCUSSION

Dietary calcium when added to a low zinc ration increased the in vitro retention of $^{65}$Zn in porcine blood cells twofold above that of the controls. The calcium effect upon cellular $^{65}$Zn uptake was not entirely due to pigs characterized by the parakeratotic syndrome, as blood cells from other pigs on the same treatment also demonstrated increased radiozinc uptake. These pigs were normal in appearance and growth but still demonstrated increased in vitro $^{65}$Zn uptake. This indicates that dietary treatment and not a stress or parakeratotic factor was responsible for the specific in vitro $^{65}$Zn behavior exhibited by

TABLE 2

*Effect of treatment on porcine blood cellular zinc levels*

| Basal diet | Basal + Zn | Basal + Ca | Basal + Zn + Ca | Basal + oil | Basal + Ca + oil |
|---|---|---|---|---|---|
| | | | *μg Zn/ml packed cells* | | |
| 8.95 ± 0.66 [1] | 8.88 ± 0.45 | 8.25 ± 0.51 | 9.30 ± 0.40 | 9.02 ± 0.51 | 8.40 ± 0.97 |

[1] Mean ± SD.

TABLE 3

*In vitro percentage uptake of radioactive minerals by porcine blood cells*

| Isotopes | Dietary treatments | | | | | |
|---|---|---|---|---|---|---|
| | Basal | Basal + Zn | Basal + Ca | Basal + Zn + Ca | Basal + oil | Basal + Ca + oil |
| $^{131}I$ | 0.39 ± 0.20 [1] | 0.57 ± 0.24 | 0.90 ± 0.28 | 0.52 ± 0.22 | 0.52 ± 0.14 | 0.86 ± 0.71 |
| $^{75}Se$ | 24.43 ± 6.60 | 25.38 ± 1.41 | 39.33 ± 18.02 | 19.66 ± 3.36 | 25.86 ± 4.84 | 31.92 ± 8.66 |
| $^{137}Cs$ | 5.56 ± 0.78 | 5.85 ± 0.56 | 7.33 ± 1.73 [2] | 5.81 ± 0.36 | 5.98 ± 1.44 | 7.95 ± 1.51 |
| $^{60}Co$ | 1.15 ± 0.35 | 1.29 ± 0.32 | 1.30 ± 0.40 | 1.28 ± 0.47 | 1.31 ± 0.57 | 1.32 ± 0.35 |
| $^{54}Mn$ | 8.54 ± 0.86 | 7.80 ± 1.36 | 9.86 ± 4.00 | 7.04 ± 1.36 | 8.88 ± 2.44 | 11.87 ± 1.52 |
| $^{59}FE$ | 4.20 ± 1.03 | 6.29 ± 2.58 | 8.20 ± 1.66 | 4.18 ± 1.73 [3] | 7.98 ± 6.75 | 6.98 ± 1.30 |
| $^{115}Cd$ | 4.90 ± 1.04 | 4.90 ± 0.45 | 6.13 ± 1.29 | 4.65 ± 0.77 | 4.80 ± 0.46 | 5.79 ± 2.00 |
| $^{64}Cu$ | 2.80 ± 0.52 | 2.67 ± 0.26 [4] | 3.26 ± 0.53 | 1.90 ± 0.39 [3] | 2.02 ± 0.20 | 3.02 ± 1.37 |
| $^{65}Zn$ | 33.95 ± 6.59 | 25.72 ± 2.77 [5] | 55.02 ± 7.97 [6] | 27.70 ± 1.70 [7] | 34.12 ± 4.05 | 51.04 ± 9.20 |

[1] Mean ± SD.
[2] Ca main effect, $P < 0.05$.
[3] Ca × Zn interaction effect, $P < 0.05$.
[4] Zn main effect, $P < 0.05$.
[5] Zn main effect, $P < 0.01$.
[6] Ca main effect, $P < 0.01$.
[7] Ca × Zn interaction effect, $P < 0.01$.

blood from pigs fed elevated calcium-zinc deficient rations. Also from these data, it appears that the in vitro technique used in this investigation could be utilized as a diagnostic tool to detect borderline zinc deficiencies which would otherwise go undiagnosed from the animals' appearance. Investigators may find this technique to be of value in studying mineral interrelationships to determine the mineral status of an animal at the initiation of an experiment.

Certain studies (19, 20) indicate that calcium interferes with zinc absorption; thus it was postulated that the stable zinc content of erythrocytes from pigs fed additional calcium would be decreased. Stable zinc content of swine blood cells was not significantly affected by treatment (table 2) and thus does not help elucidate how dietary calcium exerted its effect. However, the mean values were lower for pigs fed excess calcium.

Plasma was not assayed for stable zinc content. If dietary calcium had decreased zinc content of the plasma, then by isotope dilution an increased proportion of zinc ions entering the blood cells from swine fed a high calcium-low zinc ration would have been $^{65}Zn$. Compared with the zinc content in the plasma, an insignificant amount was added by the radioactive dose. If a large amount of zinc had been added by the dosing solution, the concentration of zinc in the plasma would be insignificant in influencing the amount of $^{65}Zn$ entering the cell. Smith [6] reported that the addition of calcium significantly depressed serum zinc levels in steers. Savage et al. (21) analyzed tissue from 4-week-old zinc-deficient and zinc-sufficient chicks and reported differences only in the zinc content of plasma, feathers, pancreas, and bone. Contrary to Smith's observations, studies by Lewis et al. (2) show that swine plasma zinc levels do not change with an increase in dietary calcium from 0.8 to 1.2%, and only increase from 48 to 74 μg/100 ml when the zinc intake is increased from 28 to 128 ppm.

The in vitro uptake of radiominerals by porcine blood cells was not affected by the

[6] Smith, W. H., T. W. Perry, M. T. Mohler, R. B. Harrington and W. M. Beeson 1964 Performance of steers fed three levels of zinc with two levels of calcium. J. Animal Sci., 23: 893 (abstract).

TABLE 4

*Effect of calcium, zinc and oil on pigs*

| | Dietary treatments | | | | | |
|---|---|---|---|---|---|---|
| | Basal | Basal + Zn | Basal + Ca | Basal + Zn + Ca | Basal + oil | Basal + Ca + oil |
| Avg initial wt, kg | 19.30 | 19.64 | 19.30 | 19.18 | 19.07 | 19.52 |
| Avg final wt, kg | 102.60 | 109.19 | 62.88 | 105.10 | 112.93 | 86.83 |
| Avg daily gain, kg | 0.74 | 0.80 | 0.39 [1] | 0.76 | 0.84 | 0.60 |
| Daily feed consumption, kg | 2.72 | 2.94 | 1.72 [1] | 2.85 | 2.65 | 2.04 |
| Kg feed/kg gain | 3.67 | 3.68 | 4.41 | 3.72 | 3.18 | 3.40 |
| Incidence of parakeratosis [2] | 0 | 0 | 3 | 0 | 0 | 1 |

[1] Ca main effect, $P < 0.05$.
[2] Four pigs/treatment.

addition of 5% corn oil. Evidence has been presented that corn oil reduces apparent calcium digestibility and increases excretion of insoluble fecal soap in sheep (22). Corn oil did not alter the calcium effect on either $^{65}$Zn uptake or growth performance in this study.

Dietary zinc decreased blood cell retention of $^{64}$Cu. Ritchie (23) reported that 100 ppm added zinc appeared to furnish complete protection against copper poisoning in the pig. Hoefer and co-workers [7] prevented parakeratosis by addition of copper (125 ppm) or iron (100 ppm) to a basal ration containing 0.5% calcium. These observations are in contrast with the work of O'Hara (10), in which zinc deficiency appeared in pigs fed a high level of copper but not in those receiving a similar diet without added copper. Research at the University of Wisconsin (7) has repeatedly shown no beneficial effect of copper in curing or preventing zinc deficiency in swine. Although $^{115}$Cd cellular activity was not affected by dietary treatment, data appear in the literature suggesting that cadmium aggravates zinc deficiency. That permanent sterility was produced in male rats by injecting a small dose of a cadmium salt, and could be prevented or at least delayed by simultaneous administration of large quantities of zinc, supports the suggestion that cadmium competes with zinc at important cellular sites (24, 25).

Blood cells from swine fed calcium-supplemented zinc-deficient rations had increased in vitro uptake of $^{131}$I, $^{75}$Se, $^{137}$Cs, $^{54}$Mn, $^{59}$Fe, $^{115}$Cd, $^{64}$Cu, and $^{65}$Zn in comparison with other treatments. This suggests that blood cells from animals fed these rations had increased permeability to all ions studied. Statistical analyses for the in vitro activity of individual radioactive minerals showed that this calcium effect was only significant for $^{137}$Cs ($P < 0.05$) and $^{65}$Zn ($P < 0.01$).

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[7] See footnote 4.

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# Effect of Chronic Ethanol Ingestion on Liver Enzyme Changes Induced by Thiamine, Riboflavin, Pyridoxine, or Choline Deficiency [1,2]

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*ABSTRACT* The effect of ethanol on riboflavin, pyridoxine, thiamine, and choline deficiencies was studied by measuring changes in rat liver dehydrogenases and by histologic assessment of fatty change in the liver. Ethanol ingestion lessened the changes induced by riboflavin deficiency. A diminution of succinic dehydrogenase (SD) activity was induced by riboflavin deficiency; however, in ethanol-fed rats this activity was significantly less diminished, and the fatty liver observed in the riboflavin-deficient animals was prevented. In contrast, the combination of ethanol ingestion and pyridoxine deficiency caused a reduction in β-hydroxybutyric dehydrogenase (HBD) activity and caused a fatty liver, whereas pyridoxine deficiency alone or ethanol feeding alone did not. Thiamine deficiency decreased the activities of HBD and lactic (LD) dehydrogenases; these depressed levels were unaffected by ethanol ingestion. The combination of choline deficiency and ethanol ingestion decreased the activities of HBD and nicotinamide-adenine dinucleotide phosphate (NADPH) dehydrogenases; however, the activity of NADPH dehydrogenase was also reduced by either choline deficiency or ethanol alone. The data suggest that ethanol ingestion is more deleterious to pyridoxine-deficient rats than to controls.

The effect of ethanol ingestion on vitamin requirements is of interest because of the frequent occurrence of vitamin deficiencies in chronic alcoholic patients (1) and the possibility that vitamin deficiencies play a role in the pathogenesis of alcoholic cirrhosis. Clinical studies suggest that chronic alcohol ingestion increases the pyridoxine requirement (2). It has been shown that alcohol intake increases the urinary excretion of thiamine and pyridoxine but does not affect choline excretion in the rat (3). Rats fed ethanol for prolonged periods developed a decrease of thiamine diphosphate in liver mitochondria and supernatant (4). Animal experiments indicate that ethanol ingestion ameliorates to some degree the changes induced by thiamine deficiency (5, 6). Klatskin et al. (7) reported evidence that ethanol ingestion increases the choline requirement of the rat.

The present study was designed to survey the effects of riboflavin, pyridoxine, thiamine, and choline deficiencies, with and without ethanol administration, on the activity of liver dehydrogenases and on liver morphology. The activities of the following dehydrogenases were assayed: succinic (SD), choline (CD), lactic (LD), isocitric (ICD), β-hydroxybutyric (HBD), and reduced nicotinamide-adenine dinucleotide phosphate (NADPHD).

## EXPERIMENTAL

### *Plan of experiments*

Four experiments, indentical in design, were carried out to investigate ethanol ingestion and liver enzyme activity in 1) riboflavin, 2) pyridoxine, 3) thiamine, and 4) choline deficiencies. In each of the experiments, young, male rats of the Wistar strain were used whose average weights at the start of the experiments were as follows: 70 g (riboflavin deficiency), 80 g (pyridoxine deficiency), 100 g (thiamine deficiency), and 150 g (choline deficiency). These weights represented the youngest

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[2] Presented in part at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, 1965, Atlantic City, New Jersey (Federation Proc., 24: 557, 1965 (abstract)).

TABLE 1

*Composition of deficiency diets*

| | Riboflavin-deficient | Pyridoxine-deficient | Thiamine-deficient | Choline-deficient |
|---|---|---|---|---|
| | % | % | % | % |
| Casein, vitamin-free | 20 | 30 | 30 | 6 |
| α-Soya protein | — | — | — | 6 |
| Lactose | — | — | — | 3 |
| Sucrose | 43 | 28 | 28 | 42.9 |
| Mannitol | 1 | — | — | — |
| Starch | 23 | 28 | 28 | — |
| Peanut oil | 8 | — | — | — |
| Cod liver oil | 2 | — | — | — |
| Corn oil | — | 2 | 2 | 4 |
| Hydrogenated cottonseed oil [1] | — | 6 | 6 | 30 |
| Cystine | — | — | — | 0.1 |
| Non-nutrient fiber | — | 2 | 2 | 4 |
| Hawk Oser salt mixture [2] | — | 4 | 4 | — |
| Wesson salt mixture [3] | 3 | — | — | 4 |
| Vitamin mixture | *mg/kg diet* | *mg/kg diet* | *mg/kg diet* | *mg/kg diet* |
| Choline dihydrogen citrate | 1,500 | 2,000 | 2,000 | — [4] |
| Inositol | 100 | 100 | 100 | 150 |
| *p*-Aminobenzoic acid | 5 | 5 | 5 | 150 |
| Nicotinic acid | — | 20 | 20 | 135 |
| Niacinamide | 20 | — | — | — |
| Menadione | 5 | 5 | 5 | 67.5 |
| Thiamine·HCl | 5 | 3 | — [5] | 30 |
| Pyridoxine·HCl | 6 | — [6] | 3 | 30 |
| Riboflavin | — [7] | 5 | 5 | 30 |
| Calcium pantothenate | 6 | 25 | 25 | 90 |
| Folic acid | 2 | 2 | 2 | 2.7 |
| Biotin | 0.1 | 0.1 | 0.1 | 0.6 |
| Vitamin $B_{12}$ | 0.02 | 0.02 | 0.02 | — |
| Ascorbic acid | — | — | — | 1,350 |
| Vitamin E | 165 | 165 | 165 | 150 |
| | *unit/kg diet* | *unit/kg diet* | *unit/kg diet* | *unit/kg diet* |
| Vitamin D | 2,000 | 20,000 | 20,000 | 3,000 |
| Vitamin A | 20,000 | 200,000 | 200,000 | 27,000 |

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[3] Wesson, L. G. Science, 75: 339, 1932.
[4] Control diet contained 20 g/kg of choline dihydrogen citrate.
[5] Control diet contained 3 mg/kg of thiamine.
[6] Control diet contained 3 mg/kg of pyridoxine.
[7] Control diet contained 5 mg/kg of riboflavin.

animals able to tolerate the combined 30% ethanol and vitamin-deficient diets beyond a few weeks. All animals were weighed twice weekly throughout the treatment period.

Fresh diet was given twice weekly; ethanol and sucrose were given in the drinking water by means of Richter tubes (8). The total number of animals entered into an experiment was divided into 5 groups, according to diet. They were maintained with these diets until manifestations of dietary deficiency or significant weight loss was evident.

The compositions of the deficiency diets are given in table 1. The pyridoxine-deficient and thiamine-deficient diets are modifications of a previously published diet (9). The riboflavin-deficient diet is a modification of the diet used by Burch et al. (10). The choline-deficient diet is that used by Grisham et al. (11).

All 4 experiments (tables 3–6) were organized according to the following plan:

Group 1 Experimental: vitamin-deficient diet ad libitum and 30% ethanol, w/v

Group 2 Vitamin-deficient pair-fed control: vitamin-deficient diet plus sucrose in isocaloric amounts

Group 3 Sucrose pair-fed control: vitamin-supplemented diet plus sucrose in isocaloric amounts

Group 4 Ethanol pair-fed control: vitamin-supplemented diet plus 30% ethanol, w/v

Group 5 General control: vitamin-supplemented diet ad libitum

### *Measurement of liver enzyme activity*

In each experiment, at the end of the treatment period, the animals were anesthetized with ether and specimens of liver tissue were obtained for the various enzyme assays and for histologic examination. Approximately 1 g of liver was taken from the liver margin uppermost in the laparotomy wound, without regard for the lobe of origin. The assays were made on the day of biopsy. Tissue for enzyme assay was quick-frozen in isopentane for 1 minute at $-70°$ and then stored at $-20°$ until assayed.

### *Biochemical assays*

The compositions of all the assay media used are shown in table 2, except for HBD.

The liver tissue for enzyme assay was homogenized in a Servall Omnimixer micro attachment. The homogenate was diluted at a ratio of 1:50, with a 1/15 M phosphate buffer (pH 7.4) containing 0.024% EDTA.

Assays for SD activity were carried out by the method of French (12). Phenazine methosulfate was used as the electron transport catalyst and the tetrazolium dye iodonitrotetrazolium (INT) was used as the electron acceptor.

The CD, LD, ICD, and NADPHD activities were measured by a similar method

TABLE 2

*Composition of assay media* [1]

| | Enzyme assays | | | | |
|---|---|---|---|---|---|
| | SD [2] | CD | LD | ICD | NADPHD |
| Phosphate buffer, pH 7.4 | 0.033 | | | | |
| Phosphate buffer, pH 7.6 | | 0.05 | | | |
| Phosphate buffer, pH 6.9 | | | 0.023 | | |
| Tris buffer, pH 7.5 | | | | 0.01 | |
| Veronal buffer, pH 7.4 | | | | | 0.03 |
| Sodium succinate | 0.05 | | | | |
| Choline chloride | | 0.033 | | | |
| Sodium lactate | | | 0.033 | | |
| Sodium isocitrate | | | | 0.003 | |
| Sodium cyanide | | $10^{-3}$ | | | |
| Manganese chloride | | | | 0.003 | |
| NADPH | | | | | $1.3 \times 10^{-3}$ |
| NAD | | | 0.007 | | |
| NADP | | | | $1.3 \times 10^{-4}$ | |
| Phenazine methosulfate | $2.7 \times 10^{-4}$ | $2.7 \times 10^{-4}$ | $2.7 \times 10^{-4}$ | | |
| Iodonitrotetrazolium | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ |
| Liver homogenate | 1/1,500 | 1/1,500 | 1/15,000 | 1/1,500 | 1/1,500 |

[1] The values of all chemicals are given as final concentrations in moles. The liver homogenate concentration is that of the final dilution. The total volume of the assay medium is 1.5 ml.

[2] Abbreviations indicate the following dehydrogenases: SD, succinic; CD, choline; LD, lactic; ICD, iso-citric; and NADPHD, reduced nitcotinamide-adenine dinucleotide phosphate.

TABLE 3

*Liver enzyme activity: riboflavin deficiency*

| Group | No. rats | Diet [1] | Avg wt at biopsy | SD | LD | ICD | HBD [2] |
|---|---|---|---|---|---|---|---|
| | | | *g* | *moles/kg protein/hr* | | *moles/kg protein/hr* | |
| 1 | 10 | Riboflavin-deficient + ethanol ad libitum | 115 | 2.26 ± 0.13 [3,4] | 11.8 ± 0.9 [5] | 0.17 ± 0.04 [6] | 10.0 ± 0.7 |
| 2 | 9 | Riboflavin-deficient + sucrose | 111 | 1.63 ± 0.18 | 12.1 ± 1.0 [5] | 0.17 ± 0.01 [6] | 8.8 ± 1.0 [7] |
| 3 | 10 | Riboflavin-supplemented + sucrose | 183 | 3.22 ± 0.22 | 15.6 ± 1.2 | 0.47 ± 0.02 | 12.8 ± 1.2 |
| 4 | 10 | Riboflavin-supplemented + ethanol | 152 | 3.90 ± 0.20 | 16.7 ± 1.1 | 0.69 ± 0.06 | 14.7 ± 1.0 |
| 5 | 10 | Riboflavin-supplemented ad libitum | 287 | 3.73 ± 0.14 | 17.1 ± 1.2 | 0.67 ± 0.05 | 15.5 ± 1.0 |

[1] Average duration of diet, 69 days; average weight of animals when started on diet, 70 g.
[2] β-Hydroxybutyric dehydrogenase (HBD): 8 rats assayed per group.
[3] Mean ± SE.
[4] SD: Significantly different compared with groups 2–5 ($P < 0.01$).
[5] LD: Significantly different compared with groups 3–5 ($P < 0.05$).
[6] CD: Significantly different compared with groups 3–5 ($P < 0.01$).
[7] HBD: Significantly different compared with groups 3–5 ($P < 0.05$).

TABLE 4

*Liver enzyme activity: pyridoxine deficiency*

| Group | No. rats | Diet [1] | Avg wt at biopsy | SD | LD | ICD | HBD [2] |
|---|---|---|---|---|---|---|---|
| | | | *g* | *moles/kg protein/hr* | | *moles/kg protein/hr* | |
| 1 | 9 | Vitamin $B_6$-deficient + ethanol ad libitum | 202 | 3.7 ± 0.4 [3] | 12.4 ± 1.6 | 1.32 ± 0.12 | 6.2 ± 0.9 [4] |
| 2 | 9 | Vitamin $B_6$-deficient + sucrose | 232 | 3.8 ± 0.6 | 12.4 ± 1.6 | 1.24 ± 0.15 | 10.6 ± 0.7 |
| 3 | 9 | Vitamin $B_6$-supplemented + sucrose | 276 | 3.8 ± 0.6 | 14.7 ± 1.8 | 1.18 ± 0.12 | 12.3 ± 0.5 |
| 4 | 9 | Vitamin $B_6$-supplemented + ethanol | 205 | 4.1 ± 0.5 | 12.0 ± 1.2 | 1.29 ± 0.14 | 13.5 ± 0.7 |
| 5 | 9 | Vitamin $B_6$-supplemented ad libitum | 492 | 3.8 ± 0.5 | 15.9 ± 2.1 | 1.27 ± 0.15 | 12.0 ± 1.3 |

[1] Average duration on diet, 138 days; average weight of animals when started on diet, 80 g.
[2] HBD: 8 rats assayed per group.
[3] Mean ± SE.
[4] HBD: Significantly different compared with groups 2–5 ($P < 0.01$).

TABLE 5
*Liver enzyme activity: thiamine deficiency*

| Group | No. rats | Diet [1] | Avg wt at biopsy | SD | LD | HBD |
|---|---|---|---|---|---|---|
| | | | *g* | *moles/kg protein/hr* | | |
| 1 | 8 | Thiamine-deficient + ethanol ad libitum | 117 | 4.0 ± 0.4 [2] | 4.8 ± 0.8 [3] | 7.6 ± 0.9 [3] |
| 2 | 8 | Thiamine-deficient + sucrose | 112 | 4.8 ± 0.4 [4] | 4.2 ± 0.8 [3] | 5.7 ± 0.4 [3] |
| 3 | 8 | Thiamine-supplemented + sucrose | 194 | 3.2 ± 0.1 | 14.3 ± 1.8 | 11.2 ± 0.5 |
| 4 | 8 | Thiamine-supplemented + ethanol | 155 | 4.0 ± 0.2 | 12.7 ± 0.7 | 13.1 ± 0.6 |
| 5 | 8 | Thiamine-supplemented ad libitum | 265 | 4.0 ± 0.3 | 14.4 ± 1.2 | 12.5 ± 0.9 |

[1] Average duration on diet, 36 days; average weight of animals when started on diet, 100 g.
[2] Mean ± SE.
[3] LD and HBD: Significantly different compared with groups 3–5 ($P < 0.01$).
[4] SD: Significantly different compared with group 3 ($P < 0.01$).

TABLE 6
*Liver enzyme activity: choline deficiency*

| Group | No. rats | Diet [1] | Avg wt at biopsy | SD | NADPHD | HBD |
|---|---|---|---|---|---|---|
| | | | *g* | *moles/kg protein/hr* | | |
| 1 | 8 | Choline-deficient + ethanol ad libitum | 229 | 2.4 ± 0.1 [2] | 3.8 ± 0.3 | 8.8 ± 1.7 |
| 2 | 8 | Choline-deficient + sucrose | 245 | 2.8 ± 0.4 | 3.8 ± 0.4 | 9.3 ± 1.8 |
| 3 | 8 | Choline-supplemented + sucrose | 241 | 2.2 ± 0.2 | 5.1 ± 0.1 [3] | 13.0 ± 0.7 [4] |
| 4 | 8 | Choline-supplemented + ethanol | 239 | 2.8 ± 0.2 | 3.0 ± 0.2 | 14.4 ± 1.2 |
| 5 | 8 | Choline-supplemented ad libitum | 389 | 2.7 ± 0.2 | 8.6 ± 0.6 | 16.8 ± 1.1 |

[1] Average duration on diet, 133 days; average weight of animals when started on diet, 150 g.
[2] Mean ± SE.
[3] NADPHD: Significantly different compared with groups 1, 2, 4 and 5 ($P < 0.02$).
[4] HBD: Significantly different compared with groups 1 and 5 ($P < 0.05$) but not significantly different compared with group 2.

using INT. The complete assay medium containing tissue homogenate was incubated for 5 minutes in a Dubnoff metabolic shaker at 38°. The reaction was stopped with 0.5 ml formaldehyde. The reduced dye was measured on a DU Beckman spectrophotometer at 490 mμ, with a slit width of 0.04 mm. A parallel blank analysis without substrate was performed in every determination. The results of the blank were subtracted from the results of the homogenate assay containing substrate to correct for nonenzymatic reduction of the dye. A molar coefficient of extinction of $1.8 \times 10^{-4}$ was used to calculate the moles of dye reduced. This coefficient was derived by calculation from our own data and also from the data of Glick and Nayyar (13). The reaction rates were linear for all 5 enzymes. Enzyme activity was proportional to enzyme concentration at the concentrations used, with the exception of CD. In the latter, very small amounts of enzyme gave disproportionately low values.

The HBD activity was assayed by the method of Lehninger et al. (14); 0.05 ml of homogenate diluted at a ratio of 1:50 was used in a final volume of 1.5 ml of the assay medium. The spectrophotometer was equipped with a water jacket to maintain a constant temperature of 38° in the cell.

Enzyme activity was calculated on the basis of protein content of the tissue. Protein was determined with the Folin phenol reagent by the method of Lowry et al. (15). All enzyme and protein measurements were made in duplicate or triplicate.

### *Histologic examination*

Hematoxylin and eosin sections were prepared from liver biopsy specimens fixed

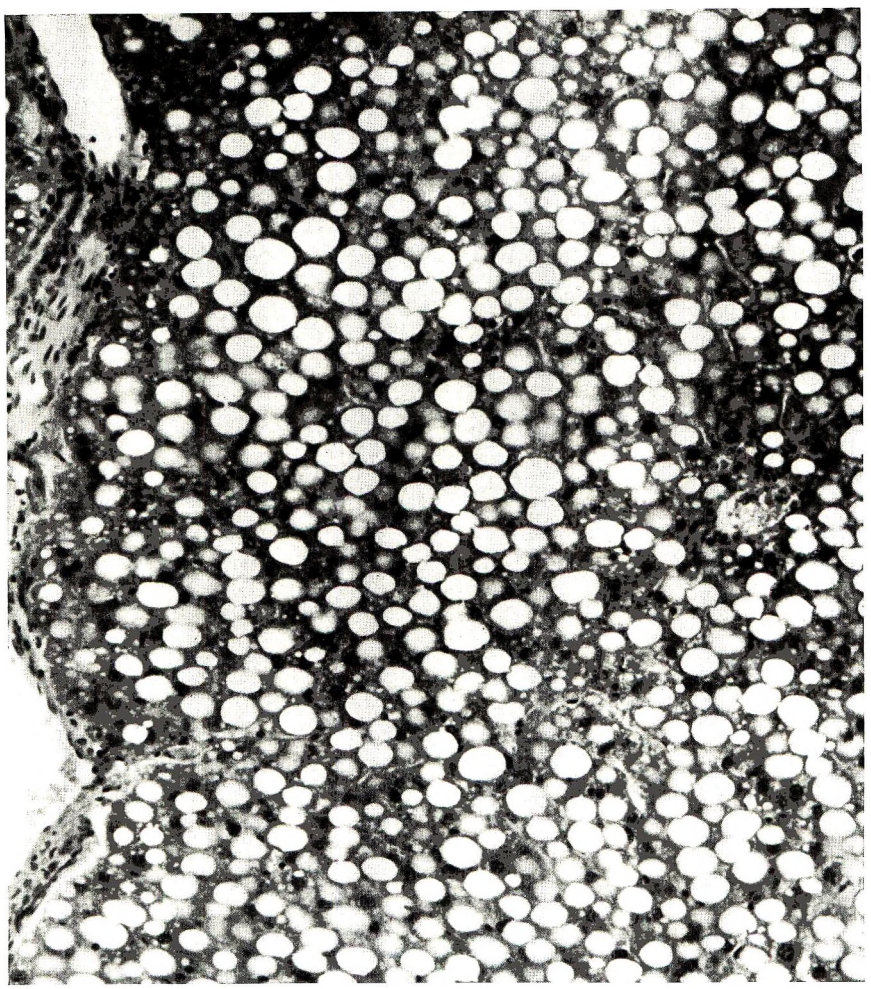

Fig. 1 Liver from a rat fed the riboflavin-deficient diet supplemented with sucrose (group 2). Note that fatty change is extensive. H & E. × 200.

in Zenker's solution and the sections were evaluated microscopically for significant fatty change. For the purpose of this study, fatty change is defined as the presence of liver cells which contain a single, large, round cytoplasmic vacuole which displaces the nucleus to the edge of the cell. In the case of the pyridoxine deficiency experiment, Oil red O fat stains were made on the livers to confirm the presence of fat in the liver cells.

## RESULTS

*Riboflavin deficiency.* When the diet was deficient in riboflavin (group 2), enzyme assays of liver showed significant decreases in activities of SD, LD, CD, and HBD (table 3). However, when the riboflavin-deficient diet was supplemented with ethanol (group 1), a sparing effect was noted in the case of SD ($P < 0.01$).

Significant fatty change in hepatic parenchymal cells was noted in 5 of the 9 rats fed riboflavin-deficient diets (group 2) (figs. 1 and 2). The lobular distribution of the fatty change was centrolobular or diffuse. In contrast, the fatty change encountered in the other rats (groups 1, 3, 4, and 5) was mild in degree and was located in the periportal region of the lobule.

The frequency of fatty change in these latter groups was as follows: 2 of 10 rats in groups 1 and 3; 0 of 10 rats in group 4; 1 of 10 rats in group 5.

*Pyridoxine deficiency.* When the diet was deficient in pyridoxine (table 4, group 2), the activities of none of the liver enzymes studied were affected except HBD. It appears that pyridoxine deficiency alone tends to lower HBD levels, and that ethanol ingestion causes a further decrease. Thus, when the pyridoxine-deficient diet was supplemented with ethanol (group 1), the HBD activity was significantly decreased. This depression is probably an indirect effect of the pyridoxine deficiency.

A striking degree of fatty change in hepatic parenchymal cells was observed in 6 of the 9 rats fed the diet combining ethanol ingestion and pyridoxine deficiency (group 1), whereas none of the rats fed the diet deficient in pyridoxine (group 2) had a significant degree of fatty change (figs. 3 and 4). These observations suggest that the deleterious effects of pyridoxine deficiency were increased by ethanol ingestion.

*Thiamine deficiency.* When the diet was deficient in thiamine (table 5, group 2), the LD and HBD activities were de-

Fig. 2 Liver from the rat pair-fed the riboflavin-deficient diet supplemented with ethanol (group 1). Compare this section with that taken from this rat's pair-fed control shown in figure 1. Note that fatty change is minimal. H & E. × 200.

pressed; these depressed values were unaffected by ethanol ingestion (group 1). An unexpected increase in SD activity was noted in the thiamine-deficient group (group 2) compared with the sucrose-fed controls (group 3). These results indicate that ethanol does not alter adversely the effect of thiamine deficiency on the liver enzymes studied.

*Choline deficiency.* The combination of choline deficiency and ethanol ingestion (table 6, group 1) decreased the activities of both NADPHD and HBD. Partial starvation alone (group 3) diminished the activity of these enzymes, but there was a further significant decrease in the ethanol-fed, choline-deficient animals (group 1). The activity of HBD tended to be reduced in

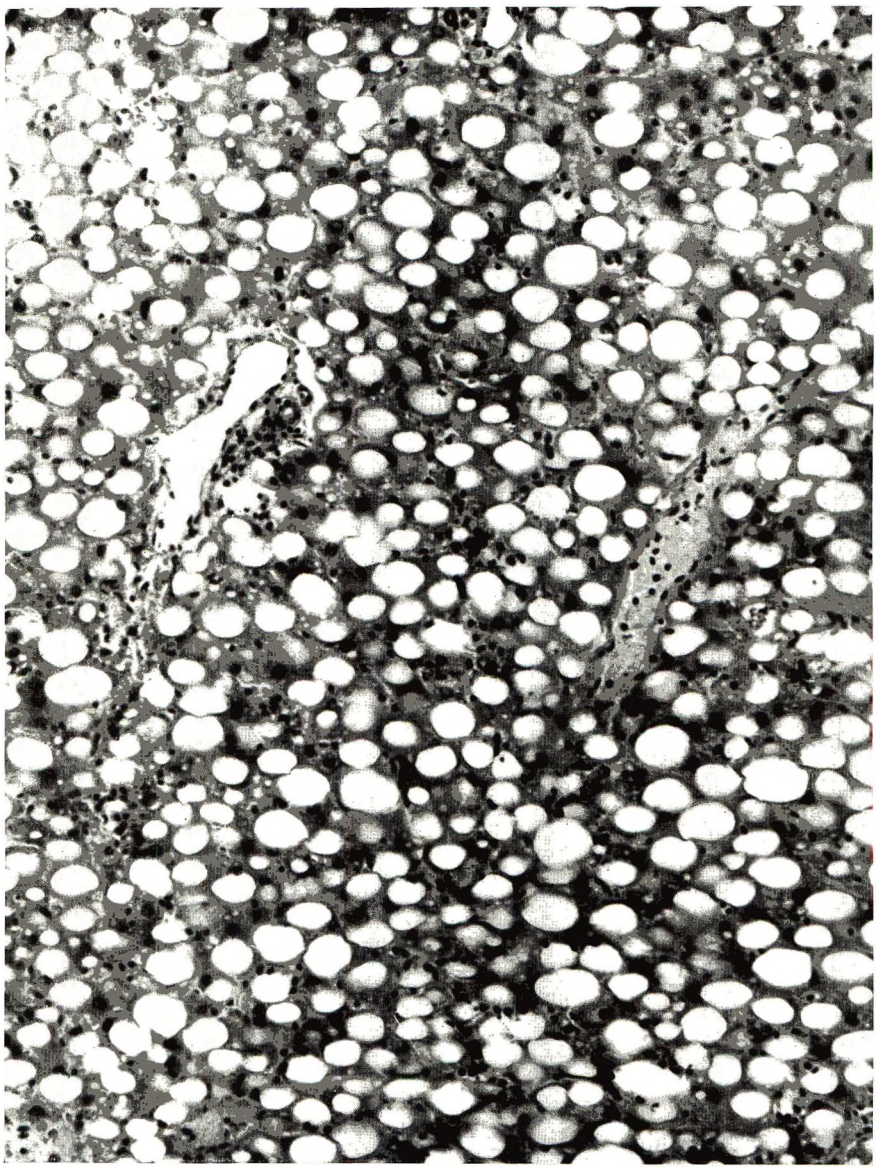

Fig. 3 Liver from a rat fed the pyridoxine-deficient diet supplemented with ethanol (group 1). Note the extensive fatty change. H & E. × 200.

choline deficiency alone (group 2) compared with that of the sucrose-fed controls (group 3), but this reduction was not statistically significant. In the case of NADPHD, however, both choline deficiency (group 2) and ethanol ingestion (group 4) significantly lowered the activity of the enzyme below that of the sucrose-fed controls. These results indicate that ethanol does not significantly alter the effect of choline deficiency on these enzymes.

Fatty change in the liver was observed in all of the rats in group 1 (choline-deficient and ethanol-fed). The fatty change was more marked in these rats than in all but two of the rats fed the choline-deficient diet alone (group 2). Figures 5 and 6 show an example of one of the more

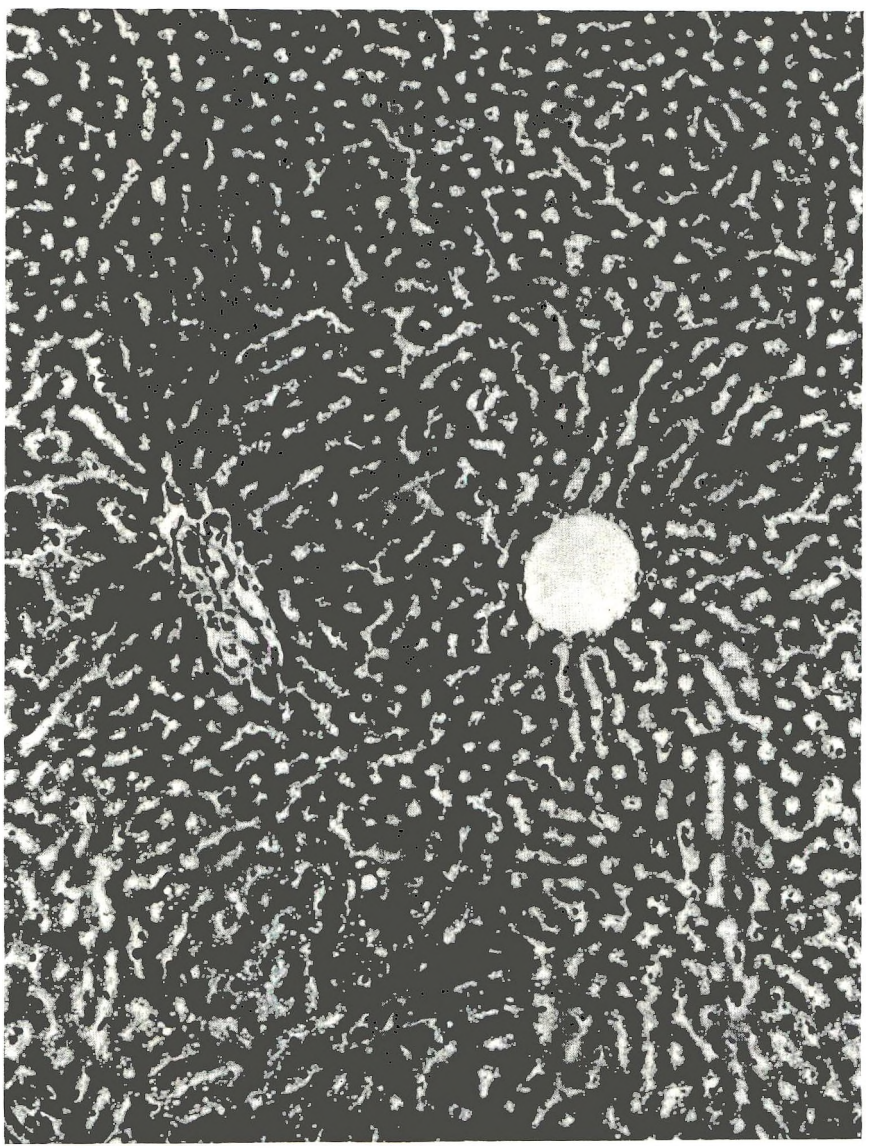

Fig. 4 Liver from the control rat pair-fed the pyridoxine-deficient diet supplemented with sucrose (group 2). Compare this section with that taken from the ethanol-fed rat shown in figure 3. Note that there is no significant fatty change. H & E. × 200.

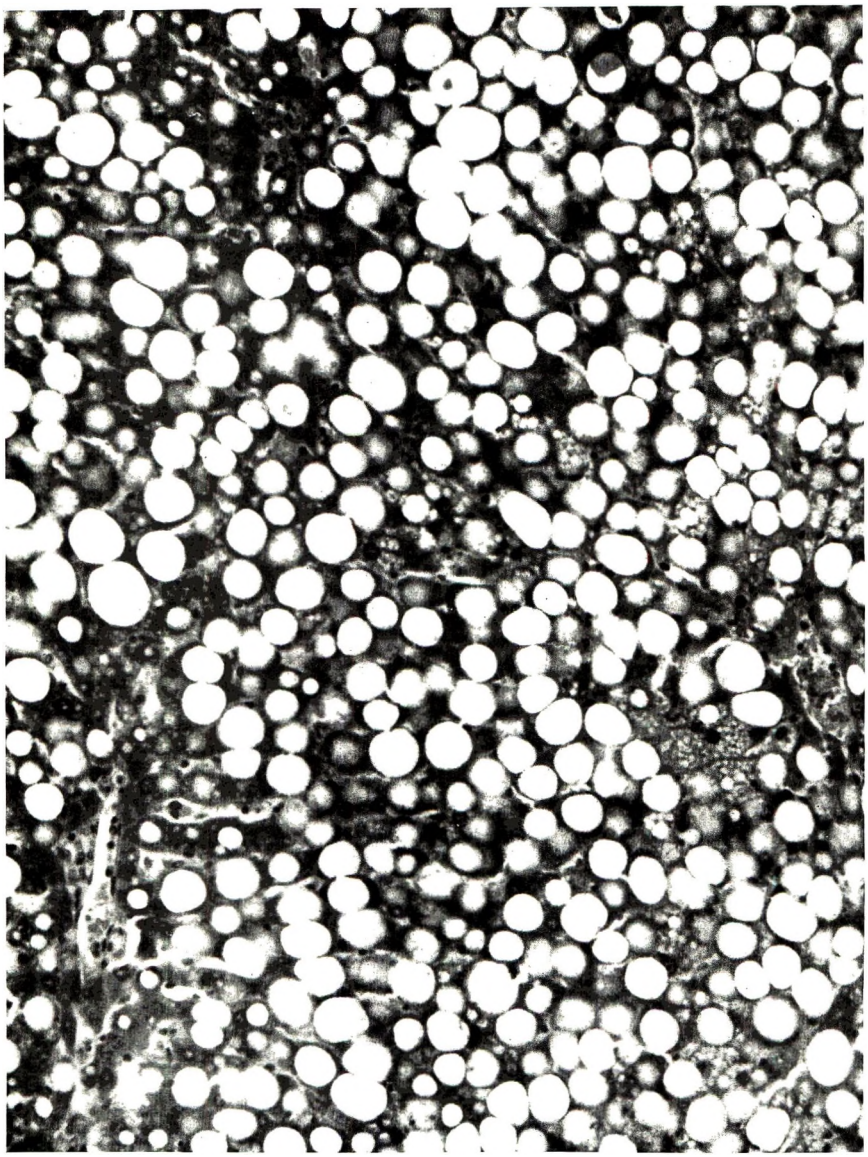

Fig. 5 Liver from a rat fed the choline-deficient diet supplemented with ethanol (group 1). Note the extensive fatty change. H & E. × 200.

striking differences. These observations suggest that ethanol increases the fatty change induced by choline deficiency, as reported by Best et al. (13) and Klatskin et al. (7).

## DISCUSSION

Ethanol feeding significantly spared the rat from the riboflavin deficiency- depressing effect on liver SD activity. The histological observations corroborated this observation in that the fatty liver commonly induced by riboflavin deficiency was not noted in the ethanol-fed riboflavin-deficient rats. Fatty liver has previously been reported in riboflavin deficient dogs (17) and swine (18). The present results imply that ethanol feeding reduces the requirement for riboflavin in the rat's diet.

In contrast, the combination of ethanol ingestion and pyridoxine deficiency caused

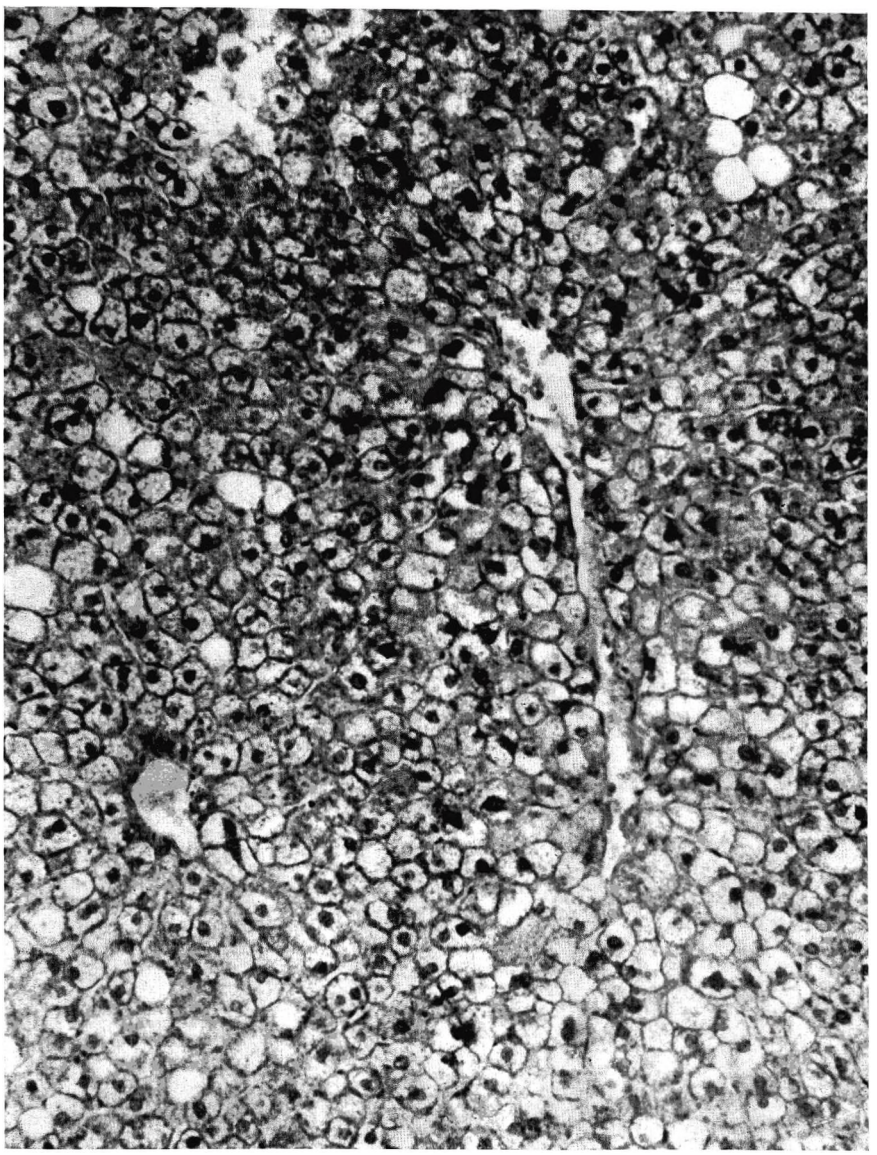

Fig. 6 Liver from the control rat pair-fed the choline-deficient diet supplemented with sucrose (group 2). Note that there are only a few large spheres of fat. Compare this section with that shown in figure 5. H & E. × 200.

fatty liver, and HBD activity was decreased, whereas pyridoxine deficiency or ethanol feeding alone did not have these effects. These data suggest that ethanol enhances the pyridoxine deficiency. This could have clinical significance in the pathogenesis of alcoholic cirrhosis, since alcoholic patients commonly have reduced blood levels of pyridoxine (1), and monkeys maintained with pyridoxine-deficient diets develop cirrhotic livers similar to those observed in alcoholic patients (19). Pasquariello et al. (2) cite evidence that there is maximal utilization of the pyridoxal-dependent enzymes concerned with conversion of tryptophan to nicotinic acid in chronic alcoholics, which may explain the vulnerability of these patients to such

disturbances as rum fits or reduced tolerance to isoniazid, which respond to pyridoxine therapy.

It is significant that the fatty liver observed in the rats fed the ethanol- and pyridoxine-deficient diet occurred in the presence of 30% dietary protein. Despite the caloric displacement of protein by ethanol, the ethanol-fed rats still consumed about 24% protein in their diet. There is evidence that a protein-rich diet does in itself increase the pyridoxine requirement (20), probably because of the importance of pyridoxine in enzyme systems affecting amino acid metabolism such as transamination. Thus, the fatty liver observed in ethanol-fed and pyridoxine-deficient rats differs from that induced in rats fed a choline-deficient ethanol diet, since the latter is prevented by increasing the dietary protein source of lipotropic amino acids such as methionine (16).

ACKNOWLEDGMENT

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# Effect of Estradiol and Testosterone on the Metabolism of Linoleic Acid in Essential Fatty Acid-deficient Rats [1]

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*ABSTRACT* The distribution of activity in carcass, liver and plasma from $^{14}C$-labeled linoleic acid given orally to 3 groups of essential fatty acid-deficient rats (male castrates (O group), castrates treated with estradiol (E), and castrates treated with testosterone (T)) was measured 6 hours after administration. More than 50% of the total incorporated radioactivity was observed in liver triglycerides and phospholipid. The specific activities of both cholesteryl ester and phospholipid were higher in plasma than in liver, but the triglycerides of liver had a much higher specific activity than the triglycerides of plasma. The cholesteryl ester in plasma and the phospholipid of liver had acquired the greatest proportion of $^{14}C$-arachidonic acid. The E group incorporated a higher proportion of radioactivity into the arachidonic acid of the liver phospholipid than the T group. Ratio of specific activity of linoleic to arachidonic was significantly lower in one of 2 liver lecithin fractions in the E group than in the T group. This fraction was richer in stearic and arachidonic acids in contrast with a less polar fraction rich in palmitic and linoleic acids. Possible implications with respect to the effects of the gonadal hormones on the conversion of linoleic to arachidonic acid are discussed.

Essential fatty acid (EFA) requirements are greater for male rats than for females (1) and male rats fed a fat-free diet showed growth depression and more severe dermal symptoms than did females (2). The reasons for this are not clear. Several lines of evidence indicate that one reason for this relatively lower resistance of male rats to the effects of an EFA deficiency may be a greater facility of the female to convert linoleic acid to arachidonic, or to conserve either or both acids when the supply is limited. Horner (3) showed that EFA-depleted, cholesterol-fed female rats had a higher proportion of dienoic and tetraenoic acids in their liver lipids than did males. Ostwald et al. (4) reported that female rats subjected to diets low in linoleic acid were better able to mobilize linoleic acid from adipose tissue than were similarly treated males.

More recent work from our laboratory (5, 6) has shown that EFA-deficient female rats and estrogen-treated, castrate male rats maintained higher concentrations of linoleate in adipose tissue and higher proportions of arachidonate in liver and plasma phospholipid (as well as in plasma cholesteryl ester) than did males or testosterone-treated castrates. This suggested that gonadal hormones may have some effect on the metabolism of essential fatty acids.

Although the biochemical consequences of an EFA deficiency and the metabolism of linoleic acid have been investigated intensely, relatively little use has been made of $^{14}C$-linoleic acid to investigate its metabolism in EFA-deficient animals (7, 8). We are now reporting the results of an experiment designed to investigate the influence of estradiol and testosterone on the incorporation of $^{14}C$-linoleic acid into the lipids of carcass, liver and plasma of EFA-deficient rats, the conversion of $^{14}C$-linoleic acid to $^{14}C$-arachidonic acid and the distribution of linoleic and arachidonic acids between liver and plasma lipid classes. The results indicate that the presence of estradiol or the absence of testosterone

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may facilitate the formation of arachidonic acid or its transfer to a more mobile form of lecithin. Observations on the specific activity of cholesteryl esters in plasma and liver indicate that the plasma may play a significant role in the esterification of cholesterol with unsaturated fatty acid.

## EXPERIMENTAL

*Animals.* Male rats of the Long-Evans strain, 6 weeks old and weighing about 155 g, were castrated and assigned to different hormone treatment groups. Within 3 days after the operation they received, by subcutaneous injection 3 times a week, 0.1 ml of either hydrogenated coconut oil or the hydrogenated oil containing 0.66 mg testosterone propionate,[2] or the hydrogenated oil containing 10 μg estradiol benzoate.[3] The groups of animals are referred to as O, oil group; T, testosterone group; and E, estradiol group, respectively. The animals were fed ad libitum a semipurified diet deficient in essential fatty acids, but containing 10% fat in the form of hydrogenated coconut oil. The diet contained 5% casein, 15% egg albumin, sucrose and the appropriate vitamins and minerals. The detailed composition of the diet has been described previously (5). After 15 weeks of this treatment, 6 animals of each group were fed linoleic acid-1-$^{14}$C and 6 hours later they were killed. Prior to autopsy the animals had been fasted overnight (14 hours), then they were allowed to eat their usual diet for one hour, and then stomach-tubed with either 1 ml or 0.5 ml/100 g body weight of safflower oil containing approximately 5 μc of linoleic acid-1-$^{14}$C. This regimen was adopted to permit efficient absorption of the linoleic acid during active food digestion and still provide animals in as uniform as possible state of alimentation.

*Preparation of tissues, extraction and analysis of lipids.* Blood was collected by open-heart puncture. The livers were weighed, frozen, lyophilized and stored at −10°. The carcasses (after removal of heart, adrenals, head and tail) and the intestines plus contents were saponified in alcoholic KOH.

The lipids of each plasma sample and each liver were extracted with ethanol followed by ethyl ether. The extracted lipids were fractionated on silicic acid columns into cholestryl esters, triglycerides, phospholipid and a fraction consisting of unesterified cholesterol, unesterified fatty acids, and mono- and diglycerides (fraction III). Total lipid, cholesterol and phosphorus were determined on the unfractionated extracts and the appropriate fractions. The fatty acid composition of the individual fractions was determined by gas-liquid chromatography (GLC) and the radioactivity of the fractions was measured as described below.

The fatty acids of each carcass were extracted from the acidified saponification mixture with petroleum ether, methylated and analyzed by GLC. The details of these procedures have been described previously (5, 6). The fatty acids of the intestines plus their contents (the gut) were similarly extracted, and their radioactivity was determined (see below).

The phospholipid fraction of the livers was combined into 2 pools for each experimental group. These phospholipid samples were then fractionated into subclasses by the method of Newman et al. (9). The phosphatidyl-choline peak (PC) was further subdivided into 2 fractions. The lecithins eluting early from the columns were called the fast-moving lecithins (F-PC) and the material eluting later, the slow-moving lecithins (S-PC). The cephalins, when analyzed by TLC contained more than a single component, whereas the lecithins appeared to be quite homogenous, except for the very early fraction which appeared to contain traces of phosphatidyl inositol or phosphatidyl serine. The fatty acid composition of the cephalins, phosphatidyl inositol, phosphatidyl serine, the 2 lecithin fractions (fast- and slow-moving), sphingomyelin and lysolecithin were determined, as were the radioactivities of phosphatidyl ethanolamine plus phosphatidyl serine, phosphatidyl inositol, and the 2 phosphatidyl-choline fractions. Sphingomyelin and lysolecithin, which represented about 10% of the total phospholipids, have not been included in this report.

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[2] USP, obtained from Nutritional Biochemicals Corporation, Cleveland.

[3] See footnote 2.

*Measurement of radioactivity.* The linoleic acid-1-$^{14}C$ [4] with a specific activity of 22.2 mc/μmole, in benzene, was evaporated under $N_2$ and diluted with safflower oil to contain either $4.6 \times 10^6$ count/min/ml or $9.5 \times 10^6$ count/min/ml. Analysis by GLC showed it to contain 75% of 18:2 acid; 89 to 92% of radioactivity recovered from the column was associated with the 18:2 mass peak. The remaining 10% was relatively evenly distributed over the chromatogram at 0.3 to 0.5 count/min/mm. The sum of the counts obtained in the effluent from the GLC column was within 2 to 3% the same as the counts of an equal aliquot placed directly on a cartridge.

The methyl esters eluted from the GLC column were collected on anthracene cartridges by means of a Packard GC Fraction Collector, Model no. 830. The following fractions were collected: the effluent from origin to, and including, 18:1 (pre-linoleic acid); 18:2 only (linoleic acid); between 18:2 and 20:4 (post-linoleic acid); 20:4 only (arachidonic acid); and past 20:4 to a time equal to that elapsed from origin to appearance of 20:4 (post-arachidonic acid). The activity in the "pre-linoleic acid" cartridge was presumed to be due to linoleic acid and possibly shorter-chain fatty acids which had become labeled by recycling of labeled acetate. The activity in the "post-arachidonic acid" cartridge was due partially to tailing of 18:2 and 20:4 and possibly to longer-chain labeled acids, although no further mass peaks have been observed. A comparison of the specific activity of the "20:4" cartridge with that of arachidic acid obtained from it by hydrogenation showed the "20:4" activity to be due to a 20 C-chain ester presumably arachidonic acid, although the presence of isomeric 20:4 acids has not been excluded. Radioactivity in any isomer other than the ω6 could have arisen only by recycling of acetate. The sum of the counts obtained from these 5 samples was approximately 15% less than the counts of an equal aliquot placed directly on a cartridge. There was less than 3% quenching of the radioactivity as determined by the use of $^{14}C$-toluene or $^{14}C$-benzoic acid as an internal standard, in toluene and on anthracene, respectively.

All measurements were made on a Packard TriCarb Scintillation Spectrometer, Model no. 314. The samples were counted either in toluene containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-4-methyl-5-phenyloxazolyl benzene (POPOP) or on an anthracene crystal cartridge (Packard TriCarb). The efficiency in toluene was 55% as determined with a Packard Standard $^{14}C$-toluene sample. No standard was available for the anthracene cartridges. Aliquots of the same sample were found consistently to count 35% lower on anthracene than in toluene when they were counted at the same voltage. All samples were counted 3 times for 30 minutes each, and the results were averaged. Since all counts within a series of replications were well within 5% of each other, no corrections for counting errors were made.

## RESULTS AND DISCUSSION

*Growth and lipid composition.* Data for body weights, liver weights and lipid composition of carcass, liver and plasma are shown in table 1. The growth of animals and their external EFA deficiency symptoms were similar to those described earlier (5). The estrogen-treated animals were smaller than either the oil-treated controls or the testosterone-treated animals, but they had enlarged livers relative to their body weights — a phenomenon we have previously interpreted to indicate a possible increased sensitivity of the EFA-deficient animals to estrogen. The carcasses of all 3 groups averaged about 10% total lipid. The levels of total lipid, phospholipid and esterified cholesterol in both liver and plasma were similar to those reported previously for 9-week EFA-deficient rats (5, 6). The level of triglycerides was much higher because of the different protocol used in the 2 experiments: The animals in the earlier deficiency study had been fasted 18 hours before killing, whereas those in the present experiment had been without food for only 6 hours and

[4] Obtained from Bio Rad Laboratory, Richmond, California. Radioautographs of chromatograms of this linoleic acid-1-$^{14}C$ on paper impregnated with mineral oil and developed with methanol: 0.01 N HCl (100:1) and on paper impregnated with olive oil and developed with ethanol:water (75:25) were provided by the manufacturer. They established radiochemical purity by these criteria.

TABLE 1

*Body and liver weights and lipid composition of carcass, liver and plasma of EFA-deficient, hormone-treated rats* [1]

| Exp. group [2] | Body wt at autopsy [3] | Liver wt | Carcass total lipid | Liver [4] | | | | Plasma | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total lipid | Cholesteryl ester | Triglyceride | Phospholipid | Cholesteryl ester | Triglyceride | Phospholipid |
| | *g* | *g* | *g/100 g* | *g/100 g* | *g/100 g* | *g/100 g* | *g/100 g* | *mg/100 ml* | *mg/100 ml* | *mg/100 ml* |
| O | 295 ± 11.0 | 9.99 ± 0.42 | 10.10 ± 0.95 | 5.04 ± 0.23 | 0.045 ± 0.007 | 0.220 ± 0.013 | 2.92 ± 0.03 | 47 ± 3.4 | 65 ± 5.5 | 162 ± 4.5 |
| E | 202 ± 6.5 | 8.75 ± 0.38 | 9.54 ± 0.66 | 4.31 ± 0.24 | 0.036 ± 0.002 | 0.138 ± 0.016 | 3.09 ± 0.07 | 34 ± 1.7 | 97 ± 29.0 | 152 ± 8.3 |
| T | 271 ± 10.0 | 8.92 ± 0.30 | 8.97 ± 0.75 | 5.28 ± 0.39 | 0.070 ± 0.008 | 0.218 ± 0.028 | 3.30 ± 0.08 | 31 ± 1.7 | 68 ± 16.0 | 115 ± 5.0 |

[1] Groups of castrate male rats after 15 weeks of hormone treatment and EFA-deficient diet.

[2] O, E, and T refer to groups of 6 animals each, that had received coconut oil, estradiol or testosterone injections, respectively.

[3] Mean ± SE.

[4] Milligrams of fraction per 100 ml plasma or grams of fraction per 100 g liver, calculated from the amount of cholesterol and phosphorus found in cholesteryl ester and phospholipid fractions, respectively, or from data obtained from GLC with help of internal standard for triglyceride fraction.

were still absorbing glyceride fatty acids. The cholesteryl ester concentrations in the livers of the T group were higher than in those of the other 2 groups (T vs. E, $P < 0.01$). This was consistent with our previous observation that EFA-deficient females or estrogen-treated castrates do not accumulate as much liver cholesteryl ester as do males or their castrate equivalents. Plasma cholesteryl ester and triglyceride concentrations were not greatly influenced by estradiol or testosterone. Phospholipid concentrations, however, were significantly lower in testosterone-treated rats than in the castrates or estrogen-injected animals (E vs. T, $P < 0.01$).

*Polyunsaturated fatty acids.* The distribution of di- and polyenoic fatty acids in carcasses, liver and plasma is shown in table 2. The mean values for carcass 18:2 acid were about 300 mg for all 3 groups. Thus, the E and T groups showed no significant difference in ability to conserve 18:2 acid after 15 weeks of receiving the linoleate-deficient diet. It has recently been shown, however, that only about half of the carcass 18:2 acid of rats fed hydrogenated coconut oil for 95 days was linoleic acid (the 9,12-isomer). The remainder consisted of the 8,11- and 6,9-isomers (10). Since in the present experiment the isomeric 18:2 acids have not been separated, the data do not indicate unequivocally whether estrogen treatment led to a greater preservation of body stores of linoleic acid than testosterone treatment.

The E group had a higher proportion (wt %) of arachidonic acid esterified with plasma cholesterol than did the T group (E vs. T, $P < 0.01$) and consequently a higher concentration (mg/100 ml plasma) of circulating cholesteryl-arachidonate. In the liver however, although the E group had a higher proportion (wt %) of cholesteryl-arachidonate than the T group, there was no difference between the 2 groups in the concentration (mg/100 g liver) of arachidonic acid esterified with cholesterol. The larger amount of cholesteryl ester (g/100 g) in the livers of the T group than of the E group (table 1) balanced the smaller percentage of cholesteryl-arachidonate.

The E group maintained a higher proportion of arachidonic acid not only in plasma cholesteryl esters but also in plasma phospholipids compared with the T group ($P < 0.01$), thus leading to a greater concentration of circulating phospholipid-arachidonate. In the liver the proportion of phospholipid-arachidonate no longer differed statistically between the groups in contrast with differences observed earlier in the EFA deficiency (5). The total amount of liver phospholipid-arachidonate also was the same for the 3 groups. The implications of these observations with respect to the formation of cholesteryl ester and to the differences between the sexes in response to an EFA deficiency are discussed elsewhere (6).

Because the animals had been fed linoleate, the plasma and liver lipid linoleate was much higher than in comparable groups of EFA-deficient animals. No differences were observed between the E and the T groups in the levels of linoleic acid in any of the plasma or liver lipid classes. The O group was consistently lower than the others. The reasons for this are unknown.

In the previous studies, 9 week EFA-deficient, testosterone-treated rats had plasma concentrations of cholesteryl eicosatrienoate and of liver phospholipid eicosatrienoate of 24% and 19.6%, respectively. In the present experiment, in which the animals had linoleic acid available for only 6 hours, comparable concentrations of eicosatrienoate were 14.5% and 13.5%. Thus, when linoleic acid is made available to EFA-deficient rats, eicosatrienoate declines rapidly in the tissue lipids. This point has been emphasized previously (5, 11).

*Distribution of radioactivity in lipid fractions.* Distribution of radioactivity in the lipid fractions of liver and plasma 6 hours after the administration of linoleic acid-1-$^{14}$C is shown in table 3. The 6-hour interval was chosen because work in other laboratories has shown 4 to 6 hours to be the time of maximal incorporation of orally administered long-chain fatty acids (12–14).

The mean activity of the dose administered to each animal was $12 \times 10^6$ count/min. Of this, $7 \times 10^6$ count/min or 58%, was found unabsorbed in the gut, and $5 \times 10^6$ count/min was recovered in the

TABLE 2

*Polyunsaturated fatty acid composition of carcass, liver and plasma lipids of EFA-deficient, hormone-treated rats* [1]

| Exp. group [2] | Carcass total lipid | | Liver | | | | Plasma | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 18:2 [3] | 18:2 | 18:2 | 20:4 | 20:3 | 18:0/16:0 [5] | 18:2 | 20:4 | 20:3 | 18:0/16:0 [5] |
| | *wt %* | *mg/carcass* | *wt %* | *wt %* | *wt %* | *wt %* | *wt %* | *wt %* | *wt %* | *wt %* |
| | | | | | Cholesteryl ester | | | | | |
| O | 1.7 ± 1.0 | 370 ± 19 | 8.3 ± 2.0 | 3.0 ± 0.6 | 3.6 ± 0.4 | | 8.3 ± 1.0 | 12.4 ± 1.6 | 16.1 ± 1.1 | |
| E | 1.5 ± 0.2 | 200 ± 20 | 19.4 ± 1.7 | 4.3 ± 0.7 | 3.4 ± 0.6 | | 13.3 ± 0.9 | 15.9 ± 1.9 | 16.3 ± 1.3 | |
| T | 2.0 ± 0.4 | 330 ± 50 | 16.1 ± 2.0 | 1.7 ± 0.7 | 4.3 ± 0.6 | | 17.0 ± 1.5 | 9.0 ± 1.0 | 14.5 ± 1.3 | |
| | | | | | Triglycerides | | | | | |
| O | | | 13.7 ± 3.7 | 0 | 1.8 ± 0.4 | | 28.5 ± 4.9 | 0 | 3.5 ± 0.6 | |
| E | | | 28.8 ± 1.9 | 1.9 ± 0.9 | 1.9 ± 0.5 | | 42.1 ± 2.0 | 0 | 3.8 ± 0.3 | |
| T | | | 28.4 ± 3.8 | 0 | 2.5 ± 1.2 | | 49.8 ± 2.1 | 0 | 3.0 ± 0.4 | |
| | | | | | Phospholipid | | | | | |
| O | | | 10.2 ± 1.0 | 13.6 ± 0.7 | 15.3 ± 0.9 | 1.86 | 16.0 ± 1.7 | 3.8 ± 1.1 | 11.4 ± 1.2 | 1.39 |
| E | | | 13.0 ± 0.7 | 15.4 ± 1.1 | 12.5 ± 0.9 | 2.19 | 20.1 ± 0.8 | 6.2 ± 1.1 | 9.4 ± 0.7 | 1.52 |
| T | | | 14.4 ± 0.7 | 13.5 ± 0.8 | 13.5 ± 0.7 | 1.73 | 22.9 ± 1.5 | 2.2 ± 0.6 | 6.7 ± 1.9 | 1.27 |

[1] As footnote 1 in table 1.
[2] As footnote 2 in table 1.
[3] Designation of fatty acids: first number is number of carbon atoms; second is number of double bonds. The isomeric composition has not been determined.
[4] Individual fatty acid as per cent of total on chromatogram.
[5] Ratio of weight % of 18:0 to 16:0 acids.

TABLE 3

*Distribution of radioactivity in the lipid classes and their PUFA of plasma and liver* [1]

| Lipid fraction | Exp. group [2] | Distribution in lipid fraction [3] | | Specific activity of fraction [4] | |
|---|---|---|---|---|---|
| | | Liver | Plasma | Liver | Plasma |
| | | *% of total* | | *count/min/μmole × 10⁻³* | |
| Cholesteryl ester | O | 0.45 ± 0.07 | 27.3 ± 5.6 | 0.90 ± 0.27 | 7.97 ± 1.9 |
| | E | 0.99 ± 0.10 | 26.7 ± 4.2 | 1.72 ± 0.23 | 7.82 ± 1.2 |
| | T | 0.53 ± 0.16 | 25.9 ± 4.5 | 0.84 ± 0.25 | 7.72 ± 0.7 |
| Triglyceride | O | 43.4 ± 5.2 | 25.5 ± 6.5 | 21.4 ± 2.4 | 5.71 ± 1.4 |
| | E | 30.9 ± 3.6 | 29.3 ± 3.0 | 18.0 ± 1.7 | 3.37 ± 1.3 |
| | T | 47.3 ± 3.8 | 34.2 ± 2.2 | 23.4 ± 3.0 | 6.38 ± 0.9 |
| Phospholipid | O | 53.9 ± 5.4 | 33.6 ± 4.2 | 1.87 ± 0.14 | 2.92 ± 0.19 |
| | E | 61.2 ± 2.6 | 25.8 ± 2.0 | 1.47 ± 0.06 | 2.04 ± 0.08 |
| | T | 47.0 ± 3.5 | 22.1 ± 1.5 | 1.41 ± 0.06 | 2.21 ± 0.08 |

[1] Groups of 6 EFA-deficient, hormone-treated rats, 6 hours after intubation with linoleic acid-1-$^{14}$C (means ± SE).
[2] As footnote 2 in table 1.
[3] Percentage of activity recovered in plasma and liver, respectively, as cholesteryl ester, triglyceride and phospholipid. In liver about 5% and in plasma about 15% of the total activity was in a fraction containing mono- and diglycerides and unesterified fatty acids.
[4] Calculated from count/min/100 ml plasma (or 100 g liver) and μmole of cholesteryl ester (or triglyceride or phospholipid)/100 ml plasma (or 100 g liver), calculated as cholesteryl-oleate, triolein and distearoyl-phosphatidyl-choline, respectively.

carcass, liver and whole blood. Total recovery of activity was 95%. Of the absorbed activity, 50% was noted in the carcass, 25% in liver, and 25% in whole blood. Plasma contained roughly 3% of the absorbed activity. No differences were observed among the groups. This distribution is similar to that observed by Reinius (15), who fed a fat-deficient rat $^{14}$C-linoleate and after 6 hours found 46% of the administered dose in the gut and 25% and 45% of the absorbed dose in the liver and carcass, respectively. Coniglio (16) fed $^{14}$C-arachidonate to rats maintained with a fat-free diet and observed that 6 hours later 76% had been absorbed, of which 6% was in the liver and most of the remainder in the carcass. The amount of activity in the blood was not measured in either of these studies.

*Distribution of radioactivity in lipid classes.* In plasma, about one-third of the activity appeared in each of the 3 lipid fractions, cholesteryl ester, triglyceride and phospholipid. The specific activity of the plasma cholesteryl ester (count/min/μmole cholesteryl ester), however, was more than twice that of the phospholipid. The O group had incorporated more activity into the phospholipid fraction than had either of the other 2 groups (O vs. T, $P < 0.05$). This is consistent with the higher plasma phospholipid level observed in the O group compared with that in the other 2 groups. Mead (17) had reported that 4 hours after feeding $^{14}$C-linoleate to adequately fed and intact rats, about 30 to 35% of the activity in plasma was in cholesteryl ester with 35 to 40% in phospholipid and about 10% in triglyceride.

In the livers, practically all of the activity appeared in the triglyceride and phospholipid fractions and less than 1% in cholesteryl ester. The specific activity of the cholesteryl ester, was, however, of the same order as that of the phospholipid. The E group had a higher proportion of activity in the phospholipid and a lower proportion in the triglyceride than did the other 2 groups (triglyceride and phospholipid, E vs. T, $P < 0.01$). The specific activity of the phospholipid was the same for the 3 groups. Coniglio (16) reported 65 to 75% of the activity of liver $^{14}$C-arachidonic acid in the phospholipid with only 1 to 2% in the cholesteryl ester fraction. Hanahan (13) also reported a greater incorporation of $^{14}$C-linoleic acid into liver phospholipid of adequately fed rats than into other fractions.

A comparison of the data for plasma and liver shows that the cholesteryl ester had a higher specific activity in plasma than in liver, whereas for the triglyceride the reverse was true. Specific activity of phospholipid tended to be higher in plasma

TABLE 4

*Incorporation of radioactivity into linoleic and arachidonic acids of liver and plasma lipids* [1]

| Lipid fraction | Exp. group [2] | % activity [3] of fraction recovered as | | | | Specific activity [4] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Liver 18:2 [5] | Plasma 18:2 | Liver 20:4 [5] | Plasma 20:4 | Liver 18:2 | Plasma 18:2 | Liver 20:4 | Plasma 20:4 |
| | | % | % | % | % | (*count/min/μmole FA*) $\times 10^{-3}$ | | | |
| Cholesteryl ester | O | 63.8 ± 3.1 | 69.9 ± 5.2 | 2.7 ± 0.5 | 6.4 ± 1.8 | 3.60 ± 1.1 | 28.4 ± 2.5 | 0.47 ± 0.02 | 1.71 ± 0.9 |
| | E | 76.4 ± 1.9 | 55.6 ± 4.6 | 2.0 ± — | 8.8 ± 1.4 | 3.73 ± 0.7 | 15.2 ± 2.2 | 0.46 ± 0.01 | 2.17 ± 0.6 |
| | T | 78.4 ± 3.3 | 71.3 ± 3.9 | 1.6 ± 0.6 | 6.3 ± 1.2 | 2.01 ± 0.6 | 11.6 ± 1.8 | 0.35 ± 0.005 | 2.35 ± 0.4 |
| Triglyceride | O | 65.3 ± 3.6 | 81.6 ± 4.3 | 1.2 ± 0.2 | 0.8 ± 0.2 | 34.3 ± 4.8 | 5.04 ± 0.8 | | |
| | E | 77.7 ± 1.5 | 82.8 ± 3.3 | 1.3 ± 0.2 | 1.1 ± 0.2 | 15.2 ± 2.0 | 1.89 ± 0.9 | | |
| | T | 82.7 ± 1.6 | 87.7 ± 1.1 | 1.2 ± 0.2 | 0.7 ± 0.1 | 23.1 ± 3.9 | 3.50 ± 0.5 | | |
| Phospholipid | O | 55.0 ± 4.3 | 64.0 ± 5.8 | 9.5 ± 1.6 | 3.7 ± 0.8 | 5.45 ± 1.5 | 6.48 ± 0.6 | 0.72 ± 0.02 | 1.56 ± 0.9 |
| | E | 56.4 ± 1.4 | 64.9 ± 1.9 | 12.3 ± 1.1 | 4.6 ± 0.6 | 3.19 ± 0.4 | 3.93 ± 0.7 | 0.66 ± 0.01 | 0.96 ± 0.4 |
| | T | 66.3 ± 2.6 | 66.8 ± 4.3 | 9.8 ± 1.4 | 4.0 ± 0.5 | 3.26 ± 0.5 | 4.40 ± 0.5 | 0.62 ± 0.02 | 2.61 ± 0.7 |

[1] As footnote 1 in table 3.
[2] As footnote 2 in table 1.
[3] Percentage of total activity recovered from GLC in cartridge, corresponding to mass peak of 18:2 and 20:4, respectively.
[4] Calculated from count/min/100 ml plasma (or 100 g liver) in lipid class, and μmole of 18:2 (or 20:4) in cholesteryl ester (or triglyceride or phospholipid)/100 ml plasma (or 100 g liver).
[5] As footnote 4 in table 2.

than in liver. In the carcass the mean of the specific activity of linoleic acid for all 3 groups was approximately $3 \times 10^3$ count/min/μmole.

*Incorporation of radioactivity into arachidonic acid.* In all fractions of both plasma and liver the major part of the radioactivity was still present as linoleic acid (table 4). Of the three lipid fractions studied, plasma cholesteryl ester and liver phospholipid had acquired the greatest proportion of activity as arachidonic acid. This corresponds to the observation of a greater proportion of arachidonic acid in plasma cholesteryl ester and liver phospholipid, respectively, than in the other fractions. The specific activity of linoleic and arachidonic acids esterified with cholesterol was higher in plasma than in liver (in E group plasma vs. liver cholesteryl ester-linoleic, $P < 0.01$, cholesteryl ester-arachidonic, $P < 0.05$), whereas the differences between plasma and liver phospholipid linoleic and arachidonic acids were on the border of statistical significance (in T group plasma vs. liver phospholipid-linoleic, $P < 0.01$, phospholipid-arachidonic, $P < 0.02$). The specific activity of linoleic acid in triglyceride was much higher in liver than in plasma. In contrast with our observations with EFA-deficient animals, Hanahan (13) reported that in adequately fed rats the specific activity of linoleic acid not only of phospholipid but also of neutral lipids was higher in plasma than in liver at all times from 1 to 16 hours. Although we measured radioactivity at only one time interval, the data from the present experiment indicate that 6 hours after refeeding of linoleic acid to EFA-deficient rats, the major part of the absorbed linoleic acid had been removed from the circulation and incorporated into liver triglyceride and phospholipid. The circulating cholesterol had become esterified with the newly available linoleic acid and newly synthesized arachidonic acid and the liver had excreted phospholipid esterified with arachidonic acid that had a somewhat higher degree of labeling than that remaining in the liver. We are aware that the intestinal mucosa might contribute to the circulating cholesteryl ester and phospholipid. Since the magnitude of this effect is unknown

we are not considering it in this discussion.

The observation that the specific activity of cholesteryl ester and of phospholipid of plasma is higher than that of the liver lends support to other evidence that a significant portion of cholesterol is esterified in plasma rather than in the liver. Glomset (18) has shown a transesterification in vitro between free cholesterol and a fatty acid derived from the β-position of plasma lecithin. Sugano and Portman (19) have come to a similar conclusion from the observation that the specific activity of plasma cholesteryl ester was higher than that of the liver cholesteryl ester for 12 hours following the injection of cholesterol-$^{14}$C.

The differences between the E and T groups in the incorporation of activity into arachidonic acid of liver and plasma lipids were less than might have been expected from the differences observed in gross composition (tables 1 and 2). Because of this apparent discrepancy, the liver phospholipids were fractionated into their subclasses and the lecithins were further separated into a stearic-arachidonic acid-rich portion, or "fast-moving" lecithin (F-PC) and a "slow-moving" (S-PC) portion rich in palmitic-linoleic acids. It has been reported previously that lecithins can be fractionated in this manner and that the resulting subfractions differ in their metabolic behavior as judged by the incorporation of $^{32}$P and $^{14}$C-fatty acids (13, 20, 21).

*Fatty acid composition of phospholipid subclasses.* Table 5 shows the fatty acid composition of the phosphatidyl ethanolamine and phosphatidyl serine, phosphatidyl inositol and the 2 lecithin subclasses. The lecithins and the phosphatidyl ethanolamine plus phosphatidyl serine fraction represented about 50% and 20% of the total phospholipid phosphorus, respectively, an observation similar to reports in the literature (20, 22). As expected, the fast-moving lecithin was richer in 16:0 and 18:2 acids. The high proportion of 20:3 acid in the phosphatidyl inositol fraction was unexpected. There was no difference between the E and the T groups in the proportion of the 2 lecithin fractions. In all phospholipid subfractions, however, the proportion of 18:0 and 20:4 acids was higher in the E than in the T group, whereas the latter was richer in the 16:0 and 18:2 acids. There was no difference between the E and the T groups in the proportion of 20:4 acid

TABLE 5

*Fatty acid composition of major phospholipid classes in livers of EFA-deficient, hormone-treated rats* [1]

| Exp. group [2] | P [3] | 16:0 [4] | 18:0 | 18:1 | 18:2 | 20:3 | 20:4 | Ratio 18:0/16:0 |
|---|---|---|---|---|---|---|---|---|
| | % | weight % | weight % | weight % | weight % | weight % | weight % | weight % |
| | | | | Phosphatidyl ethanolamine + phosphatidyl serine | | | | |
| O | 17.1 | 15.9 | 26.0 | 16.4 | 7.8 | 9.7 | 17.8 | 1.63 |
| E | 19.8 | 15.0 | 28.4 | 14.8 | 9.8 | 8.2 | 16.8 | 1.89 |
| T | 19.4 | 18.4 | 24.2 | 14.4 | 9.4 | 10.0 | 15.2 | 1.31 |
| | | | | Phosphatidyl inositol (+fast-moving lecithin) [5] | | | | |
| O | 16.0 | 11.4 | 36.2 | 8.4 | 4.6 | 19.8 | 16.4 | 3.17 |
| E | 14.3 | 10.7 | 38.5 | 7.6 | 4.3 | 18.8 | 17.0 | 3.60 |
| T | 18.6 | 14.5 | 28.8 | 10.0 | 6.6 | 17.5 | 12.6 | 1.99 |
| | | | | Fast-moving lecithin | | | | |
| O | 34.6 | 15.5 | 27.5 | 13.5 | 11.6 | 16.4 | 12.3 | 1.77 |
| E | 34.2 | 14.5 | 30.3 | 11.6 | 13.7 | 13.5 | 13.9 | 2.09 |
| T | 35.4 | 22.2 | 22.7 | 13.2 | 14.6 | 10.6 | 8.1 | 1.02 |
| | | | | Slow-moving lecithin | | | | |
| O | 17.2 | 25.0 | 25.0 | 16.8 | 14.2 | 7.4 | 4.7 | 1.00 |
| E | 17.5 | 19.6 | 25.2 | 14.0 | 18.2 | 8.6 | 9.8 | 1.28 |
| T | 13.0 | 26.2 | 20.6 | 14.8 | 17.0 | 7.7 | 4.9 | 0.79 |

[1] As footnote 1 in table 1. The composition of 5% each of sphingomyelin and lysolecithin is not presented here.
[2] As footnote 2 in table 1.
[3] Percentage of total phosphorus in phospholipid.
[4] Small amounts of myristic, palmitoleic and higher fatty acids are not presented here.
[5] Our method does not separate phosphatidyl inositol completely from lecithin.

which had been replaced by 20:3 acid. Previous work from our laboratory (23, 24) has shown that a sex difference in the distribution of stearic and palmitic acids occurred in the lecithins with a "male" pattern of phosphatidyl choline high in 16:0 and 18:2 acids and a "female" pattern high in 18:0 and 20:4 acids. The results of the present experiment indicate that for EFA-deficient animals, the E group still maintained a more pronounced female pattern in all the phospholipid subfractions with the exception of the cephalins, whereas the T group had the more pronounced male pattern.

*Incorporation of radioactivity in phospholipid subclasses.* Table 6 shows the distribution of radioactivity among the phospholipid subclasses, and their component linoleic and arachidonic acids. The lecithins contained about 70% of the total phospholipid radioactivity, corresponding to the higher linoleic acid content as compared with other classes. For each of the 3 groups, the specific activity (count/min/μmole phosphatidyl choline) of the 2 lecithin fractions was similar, indicating that, in contrast with work quoted previously, their turnover was similar under the specific conditions of this experiment. The specific activity of both lecithins tended to be greater in the E group than in the T group. The greater specific activity of the lecithins over that of the cephalins and phosphatidyl-inositol resembles the observation of Lee (25) that the order of incorporation of $^{32}P$ into mouse liver phospholipid after 8 hours was phosphatidyl choline > phosphatidyl ethanolamine > phosphatidyl inositol.

In all fractions, most of the activity was still present as linoleic acid and in most fractions the T group tended to have more activity in linoleic acid than did the E

TABLE 6

*Incorporation of radioactivity into linoleic and arachidonic acids of liver phospholipid classes from EFA-deficient, hormone-treated rats* [1]

| Exp. group [3] | Phospholipid classes | | 18:2 [2] and 20:4 acids | | | | |
|---|---|---|---|---|---|---|---|
| | Distribution among classes [4] | Specific activity [5] | Activity in class recovered as [6] | | Specific activity [7] | | Ratio of count/min/μmole 18:2/20:4 |
| | | | 18:2 | 20:4 | | | |
| | *% total phospholipids* | *count/min/μmole × 10⁻³* | *%* | *%* | *count/min/μmole × 10⁻³* | | |
| | Phosphatidyl ethanolamine + phosphatidyl serine | | | | | | |
| O | 14.2 | 1.82 | 54.4 | 15.0 | 4.7 | 0.60 | 7.7 |
| E | 18.8 | 1.78 | 54.5 | 14.4 | 3.5 | 0.60 | 5.8 |
| T | 16.8 | 1.29 | 64.2 | 14.6 | 3.1 | 0.50 | 6.3 |
| | Phosphatidyl inositol (+ fast-moving lecithin) | | | | | | |
| O | 9.0 | 1.71 | 43.5 | 16.8 | 5.0 | 0.55 | 9.0 |
| E | 7.2 | 1.19 | 42.1 | 20.3 | 3.6 | 0.49 | 7.3 |
| T | 15.8 | 1.55 | 57.4 | 16.0 | 4.1 | 0.66 | 6.2 |
| | Fast-moving lecithin | | | | | | |
| O | 48.4 | 3.39 | 59.4 | 10.2 | 6.2 | 1.04 | 6.0 |
| E | 45.5 | 2.83 | 60.4 | 13.3 | 4.4 | 1.09 | 4.1 |
| T | 50.0 | 2.17 | 74.6 | 8.2 | 3.9 | 0.38 | 10.3 |
| | Slow-moving lecithin | | | | | | |
| O | 23.4 | 3.90 | 62.8 | 6.8 | 6.1 | 2.16 | 2.8 |
| E | 25.5 | 3.05 | 65.8 | 8.6 | 3.9 | 1.05 | 3.7 |
| T | 17.4 | 2.31 | 68.4 | 6.1 | 3.3 | 1.13 | 2.9 |

[1] As footnote 1 in table 3; averages of 2 pools of 3 rats each.
[2] As footnote 4 in table 2.
[3] As footnote 2 in table 1.
[4] Percentage in the class of total count/min in phospholipid.
[5] Calculated from count/min in class/100 g liver, and μmole of class/100 g liver, assuming the distearoyl derivative.
[6] As footnote 3 in table 4.
[7] Calculated from count/min/100 g liver in 18:2 or 20:4 of the class, and μmole of 18:2 or 20:4/100 g liver in the class.

group, whereas the reverse was true for arachidonic acid. More significantly, the ratio of the specific activities (count/min/μmole 18:2 to count/min/μmole 20:4) indicate that 6 hours after feeding linoleic acid the fast-moving lecithin of liver contained relatively more labeled 20:4 acid compared with its precursor, 18:2 acid in the E than in the T group. This may or may not reflect the rate of conversion of linoleic to arachidonic acids in these animals, but it does suggest that estradiol has an effect on the 20:4 acid content of the lecithins. In combination with the tendency of the E group to incorporate more activity into total liver phospholipid (table 3) and into phospholipid-arachidonic acid (table 4), and to have a higher specific activity in one of the lecithin fractions (table 6), it appears that estradiol may facilitate the conversion of linoleic to arachidonic acid or if not the conversion, it may enhance the transfer of arachidonic acid to the more mobile liver and plasma lecithins. The apparent ability of estradiol to influence the metabolism and distribution of arachidonic acid in these animals may be related to the greater resistance of female rats to EFA deficiency symptoms as compared with males.

The 0 group, i.e., castrate males without hormone treatment, was included in the experimental design to decide whether differences observed between the E and the T group were due to a positive action of estradiol or to an inhibition by testosterone. Previous work with hormone-treated castrate rats fed adequate diets (23) or EFA-deficient rats (6, 26) suggested that the effect of estradiol is a positive one. In the present experiment the data for the O group did not resemble consistently those for either the E or the T group. Good evidence exists for an interrelationship between the metabolism of adrenocortical and of estrogenic hormones, leading, for instance, to a competitive inhibition between cortisol and estrogen (27, 28), and a stimulation of pituitary ACTH by estradiol (29). The behavior of our O group animals may therefore reflect a modified action of adrenocortical hormones, caused by the absence of gonadal hormones. The question of whether the differences we have observed between the E and T group are due to the presence of estradiol on the absence of testosterone remains open.

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# Secondary Folate Deficiency Induced in the Rat by Dietary Iron Deficiency [1]

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*ABSTRACT* The effects of iron deficiency on folate metabolism in rats were studied. Dietary iron deficiency was induced in 18-day-old male rats, following which the animals developed the expected hypochromic, microcytic anemia. In addition, notwithstanding that the diet contained adequate folate, iron deficiency resulted in biochemical and morphological changes of folate deficiency such as increased urinary excretion of formiminoglutamic acid, decreased serum folate and megaloblastoid dysplasia of the bone marrow elements. In a second experiment, iron deficiency was shown to result in a decreased activity of the enzyme glutamate formimino-transferase isolated from livers. It was concluded that a secondary folate deficiency can be induced by a dietary deficiency of iron and that this defect in folate metabolism is related to the decreased activity of the enzyme formimino-transferase whose optimal activity is dependent on iron.

The preponderance of evidence suggests that folic acid deficiency is extremely difficult to produce in mammals unless a folic acid antagonist is administered (1) or iodinated casein (2), intestinal germicidal substances (3), a high level of methionine (4) or vitamin C deficiency (5) is included with a diet deficient in folic acid.

The relationship between iron deficiency and folic acid deficiency signs (namely, megaloblastosis, increased FIGlu excretion, etc.) is a relatively unexplored area of research. It has generally been believed that iron deficiency merely concealed the hematological changes of a concomitant folic acid deficiency and that the bone marrow and peripheral blood changes were predominantly those of iron deficiency; that is, microcytosis and hypochromia (6, 7).

However, increased urinary excretions of formiminoglutamic acid (FIGlu), a biochemical sign attributed to folic acid deficiency, has been observed in patients with iron deficiency (8) and recently Chanarin et al. (9) presented evidence suggesting that iron deficiency may, in fact, produce a defect in the utilization of folic acid. Our own preliminary studies indicate that iron deficiency does, in fact, result in an increased excretion of urinary FIGlu in experimental animals [2] and in humans.[3]

The studies of Chanarin (9) and our own preliminary results (10) strongly suggest a more fundamental role of iron deficiency in folate metabolism.

The mechanisms by which iron deficiency may result in folic acid deficiency signs may be related to the integrity, or loss thereof, of those enzyme systems involved in the transfer of one-carbon units to tetrahydrofolic acid. Vitale et al. (10) in a preliminary communication indicated that the enzyme(s), glutamate formimino-transferase, involved in the transfer of the formimino group to tetrahydrofolic acid is (are) iron-dependent. Thus the increase in urinary formiminoglutamic acid in iron-deficient patients may be the result of a loss in the activity of this (these) enzyme system(s).

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TABLE 1

*Schedule for killing rats after various dietary periods*

| Days (after diet change) | No. of rats killed | | | |
|---|---|---|---|---|
| | Continued with control | Diet of rats after 7 weeks of iron deficiency | | |
| | | Continued without Fe [1] | Continued with Fe [2] | Continued with Fe, without FA [3] |
| 5 | | 3 | 4 | 4 |
| 8 | | | 4 | 4 |
| 12 | | 3 | 4 | 4 |
| 15 | | | 4 | 4 |
| 19 | 4 | 3 | 4 | 4 |
| 22 | | | 4 | 4 |
| 26 | | 3 | 4 | 4 |
| 29 | | | 4 | 4 |
| 33 | 4 | 3 | 4 | 4 |

[1] Group continued with iron-deficient diet.
[2] Group repleted with iron.
[3] Group repleted with iron but fed folate-deficient diet.

The following studies deal with the effects of iron deficiency on folate metabolism in rats.

## METHODS

Two experiments were carried out using 18-day-old male albino rats obtained from the Charles River Breeding Laboratories, Wilmington, Massachusetts. All animals were housed in individual cages and watered and fed ad libitum. The basic diet, without added folic acid or iron, consisted of the following ingredients per 100 g: glucose, 65.8; choline chloride, 0.5; casein (vitamin-free), 20.0; corn oil,[4] 10; cod liver oil, 1.0; salt mixture (11), 2.2; and 0.5 of a vitamin mixture (12). The control, or supplemented diets, contained 1 mg of folic acid (PGA) and 120 mg of iron ($FeSO_4 \cdot 7H_2O$)/kg of diet.

*Experiment 1.* Eighteen rats were fed the control diet (supplemented with iron and folic acid) and 112 rats were fed the iron-deficient diet. Five control rats were killed at zero time and at 35 days. Five rats from the iron-deficient group were killed weekly for a period of 5 weeks. After 5 weeks the iron-deficient group was divided into 3 groups as follows: 15 rats were continued with the iron-deficient diet; 36 rats were fed a diet containing iron (control diet) and a third group of 36 rats was fed a diet supplemented with iron but deficient in folic acid. The schedule of killing following the change in diet is illustrated in table 1. The entire experiment lasted 68 days.

At the time of killing, the animals were anesthetized with ether and blood was drawn aseptically from the abdominal aorta. The following determinations were made: hemoglobin (13), hematocrit by a microcapillary method, serum iron and total iron-binding capacity (14), urinary folate (15), urinary formiminoglutamic acid (24-hour urine collection prior to killing) (16), and serum folic acid (15). Autopsy specimens were preserved in 10% neutral formalin for light microscopic morphologic study. Sections of liver and spleen were stained with hematoxylin and eosin and with Gomori's ferrocyanide stain for iron. The proximal approximate one-third of the small intestine was opened, spiralled, and was stained with Alcian blue, hematoxylin and eosin. The animals were weighed twice weekly. At 40 days and thereafter, bone marrow aspirates were taken from the upper femur with a syringe containing 3% ethylenediaminetetraacetic acid. Smears therefrom were stained with Wright's stain.

*Experiment 2.* Two groups of 48 rats each were fed either the control diet or the diet low in iron. Twelve rats from each group, control and iron-deficient, were killed at weekly intervals and the determinations listed in experiment 1 were also performed. In addition, the livers of the animals were pooled at the end of the first, second and fourth week according to the 2 groups, and the enzyme glutamate formimino-transferase was isolated and assayed for its activity by the method of Tabor and Wyngarden (16).

## RESULTS

*Experiment 1.* Figure 1 illustrates the growth curves for the animals in the various groups. Iron-deficient animals gained less weight than the control animals. However, iron-deficient rats attained weights comparable to control weight after being given supplements of dietary iron; this was equally true for animals repleted with a diet containing iron but low in folate.

[4] Mazola, Corn Products Company, New York.

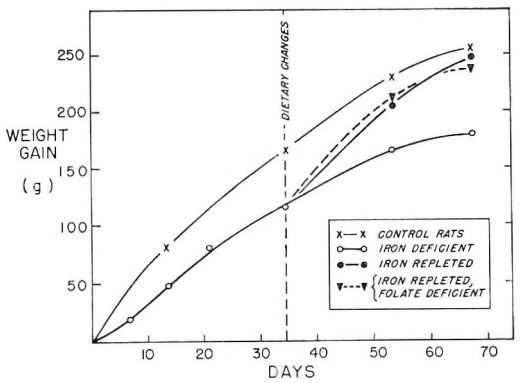


Fig. 1 Growth curves of control, iron-deficient and iron-repleted rats.

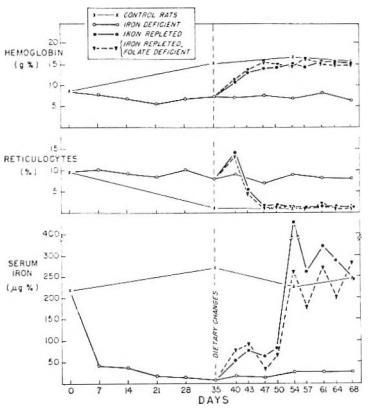


Fig. 2 Hemoglobin, reticulocytes and serum iron in control, iron-deficient and iron-repleted rats.

Figure 2 illustrates that animals fed the iron-deficient diet had decreased hemoglobin levels, an elevated reticulocyte count and decreased serum iron levels. Dietary iron, with and without dietary folate, when fed to iron-deficient rats resulted in an additional reticulocyte response which was followed by a return of the hemoglobin, serum and reticulocyte count to control values.

Figure 3 illustrates the changes in the mean cell volume, serum folate, and urinary FIGlu in the various groups. The mean red cell volume fell from control values of approximately 65 $\mu^3$ to 39 $\mu^3$ in iron-deficient rats. The serum folate levels also decreased in the iron-deficient group from approximately 120 mμg/ml to 60 mμg/ml at 35 days. The decrease in serum folate in the iron-deficient groups was accompanied by an increase in urinary FIGlu excretion. With iron repletion, the serum folate returned to normal values and FIGlu excretion returned to near-normal levels. The 24-hour urinary FIGlu concentrations showed considerable variations. We cannot explain these variations but believe that they are biological rather than methodological in origin.

Figure 4 illustrates that in iron-deficient rats, urinary folate excretion, relative to control values, is increased as is the urinary FIGlu excretion; also that after the animals were fed an iron-containing, folate-deficient diet, the urinary excretion of folate fell to below control levels and urinary FIGlu remained elevated; in this group, the serum folate (fig. 3) remained low.

At autopsy, the rats fed an iron-free diet were readily distinguishable from others by the fact that their feces were light yellow in contrast with the darker yellow or brown of the rats eating an iron-containing diet.

No group differences at the various killing dates could be observed in the upper small intestine: the parameters used were

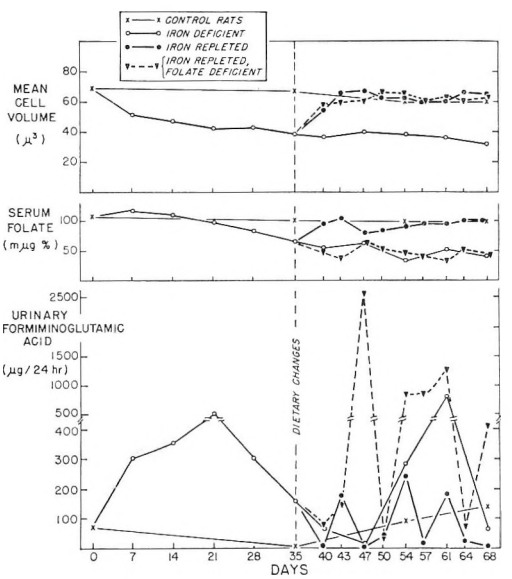


Fig. 3 Mean red cell volume, serum folate and 24-hour urinary formiminoglutamic acid of control, iron-deficient and iron-repleted rats.

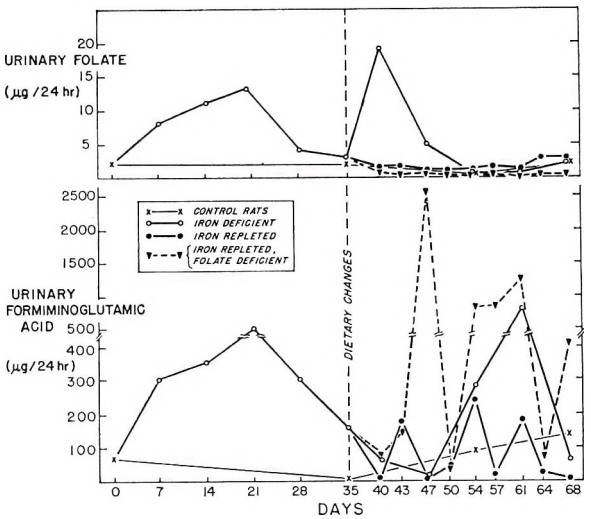


Fig. 4 Urinary folate and urinary formiminoglutamic acid, per 24-hour period, excretion in control, iron-deficient and iron-repleted rats.

villar size, gland depth, appearance of epithelial cells or infiltrate in the lamina propria.

No stainable iron was noted in the spleen or liver of rats eating the iron-free diet. Starting from virtually none at 5 weeks, the spleens of the control rats showed progressively increasing amounts of stainable iron. The spleens of the iron-repleted rats showed a similar increase starting at the ninth day of repletion; the folate content of the diets did not cause any difference in this phenomenon among the 2 iron-repleted groups.

The livers of the iron-deficient rats showed no inflammatory changes for the first 3 weeks. Thereafter approximately one-half of such rats, regardless of repletion, if any, showed significantly greater periportal mononuclear infiltrate than did rats never iron-depleted.

An attempt was made to determine the degree, if any, of megaloblastic maturation in bone marrow aspirates. No attempt was made to grade the degree of megaloblastosis when considered present. A bone marrow was considered to show megaloblastoid maturation when either the cytoplasmic-nuclear maturation of red cell precursors was dissynchronous and orthochromatic and polychromatic red cells showed an opened, loosely woven nuclear chromatin pattern rather than a dense, closely packed chromatin pattern, or when giant band cells or giant metamyelocytes were present. Using this rather loose definition of megaloblastosis, the bone marrows of ten out of 15 iron-deficient rats appeared megaloblastoid. Megaloblastic-like changes were observed in only two of the 36 iron-repleted animals, whereas iron repleted, folate-deficient and control rats maintained what we considered to be a normoblastic type of maturation.

Figure 5 shows a megaloblastoid bone marrow of a rat fed an iron-deficient diet for 40 days.

Also, the animals fed the low iron diet were anemic, had low serum iron levels, had marked decreases in the percentages of iron saturation, and grew less well compared with rats fed the control diet (table 2). The serum folate decreased and was accompanied by an increase in the urinary excretion of FIGlu.

Figure 6 illustrates the activity of the enzyme glutamate formimino-transferase from the combined livers of rats in the 2 groups after 2 and 4 weeks. The activity of this enzyme system was diminished in the livers from iron-deficient rats. The decrease in enzyme activity of iron-deficient livers was not marked at the end of one week but was more pronounced by the

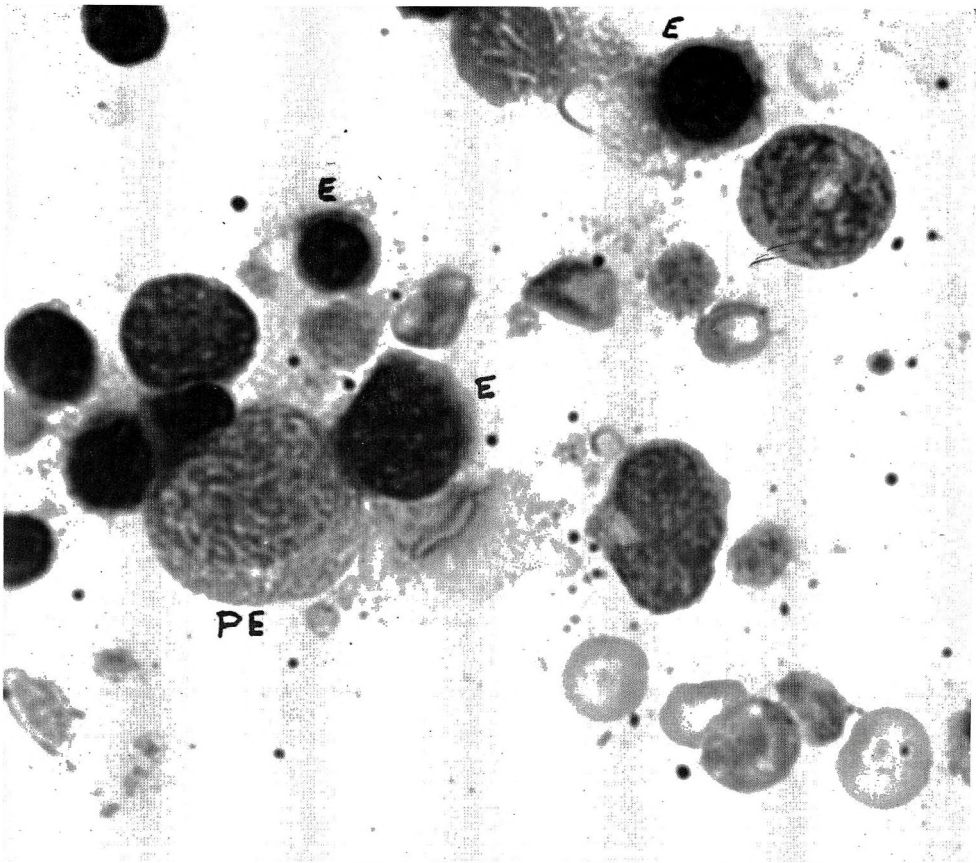


Fig. 5 Bone marrow aspirate taken from iron-deficient rat (after receiving diet for 40 days), showing megaloblastoid maturation of red cell series. *E* = polychromatic and orthochromatic erythroblasts with size variation and "vacuolated" appearing nuclei. *PE* = megaloblastoid proerythroblast.

TABLE 2

*Some effects of iron deficiency in male rats (exp. 2)*

| Group [1] | Control | | | | Iron-deficient | | | | P values [2] |
|---|---|---|---|---|---|---|---|---|---|
| Weeks | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | |
| Wt gain, g | 69 | 101 | 128 | 173 | 57 | 63 | 104 | 126 | $< 0.01$ |
| Hemoglobin, g% | 11.8 | 12.6 | 12.8 | 13.9 | 5.8 | 6.6 | 5.7 | 5.6 | $< 0.01$ |
| Hematocrit, % | 38 | 38 | 39 | 41 | 19 | 23 | 21 | 20 | $< 0.01$ |
| Serum iron, $\mu$g% | 294 | 246 | 336 | 322 | 49 | 43 | 25 | 37 | $< 0.01$ |
| T.I.B.C.,[3] $\mu$g% | 735 | 664 | 719 | 661 | 758 | 629 | 677 | 750 | ns |
| FIGlu, $\mu$g/24 hr | 69 | 165 | 119 | 59 | 72 | 169 | 361 | 423 | $< 0.05$ [4] |
| Serum folate, m$\mu$g/ml | 106 | 81 | 136 | 90 | 104 | 96 | 63 | 58 | $< 0.05$ [4] |

[1] Twelve rats/group killed each week.
[2] Control vs. iron-deficient.
[3] Total iron-binding capacity.
[4] Urinary FIGlu and serum folate significantly different at third and fourth weeks.

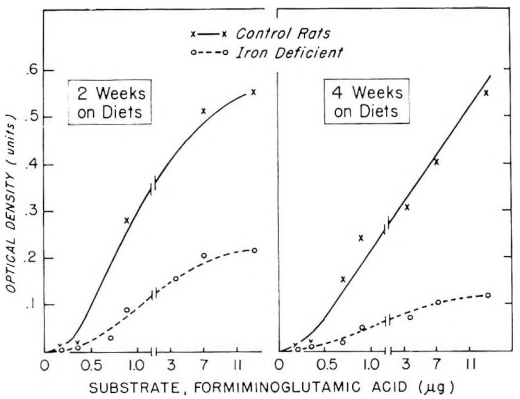


Fig. 6 Glutamate formimino-transferase activity of livers from control and iron-deficient rats (activity per gram of liver tissue).

second week. An additional decrease in the activity of the enzyme was observed at the end of 4 weeks.

## DISCUSSION

The results of these experiments support the view that iron deficiency does result in a defect of folic acid metabolism. Folic acid is known to be involved in the synthesis of nuclear material, deoxyribonucleic acid and ribonucleic acid, by virtue of its role in the transfer of one-carbon units (17). The major pathways of one-carbon transfer are either by the reaction histidine to glutamic acid, in which the intermediate formiminoglutamic acid loses the formimino group to form $N^5$-formiminotetrahydrofolate, or via the reaction in which serine is converted to glycine and the carbon removed unites with THF to form $N^{5\text{-}10}$-methylene THF.

The increase in FIGlu excretion in animals fed low folate diets was not associated with any adverse effects. This increase in FIGlu excretion without concomitant defects in growth or morphology may have several explanations. In situations where the diet is devoid or low in folate, sufficient folic acid may be supplied by the intestinal flora to meet body needs. This implies that the transfer of the formimino group of FIGlu occurs but at a much reduced rate with FIGlu excretion elevated. A second possible explanation is that in the pyridoxine-dependent system the transfer of the carbon unit from serine becomes more efficient, meeting the body needs for one-carbon units. Again, this second explanation implies that non-dietary folate is supplied from within the body.

However, in iron deficiency, the high excretion of urinary FIGlu is important in that the enzyme systems required for one-carbon transfer presumably require iron for their optimal activity or formation, or for both. Defects in growth and nuclear metabolism would be expected to occur in the absence of one-carbon transfer. This explanation implies that either the enzyme system involved in transfer of the serine carbon is iron-dependent or that this system is relatively unimportant as compared with the glutamate formimino-transferase system.

The metabolic pathways discussed are illustrated in figure 7. Thus, in iron deficiency an increase not only of urinary FIGlu excretion might be expected, but of folate excretion as well, namely, urinary folate.

It is evident that the low serum folate level and high urinary FIGlu excretion, at least in the rat, are not necessarily of biological significance in certain experimental conditions; rather they reflect dietary folate intake or availability.

It is our impression that "folate deficiency" is generally assumed to be dietary in origin. We believe, rather, that the manifestations of folate deficiency should be considered from the viewpoint of a balance of at least 3 factors: 1) supply: influenced by dietary intake, bacterial synthesis (18) and possibly biosynthesis (19); 2) demand: influenced by bacterial utilization (18) and rate of deoxyribonucleic and ribonucleic acid synthesis (growth, tumor growth, pregnancy, increased hematopoiesis, etc.); and 3) activity of enzymes and presence of factors involved in folate metabolism.

Recently, Arakawa et al. (20) reported a congenital defect in formimino-transferase of a liver in an 8-month-old female child who showed evidence of folate deficiency (20); this is an example of factor 3 above. Finally, the results of the present study indicate that the signs usually attributed to folate deficiency (megaloblastoid dysplasia, increased urinary FIGlu excretion and decreased serum folate levels)

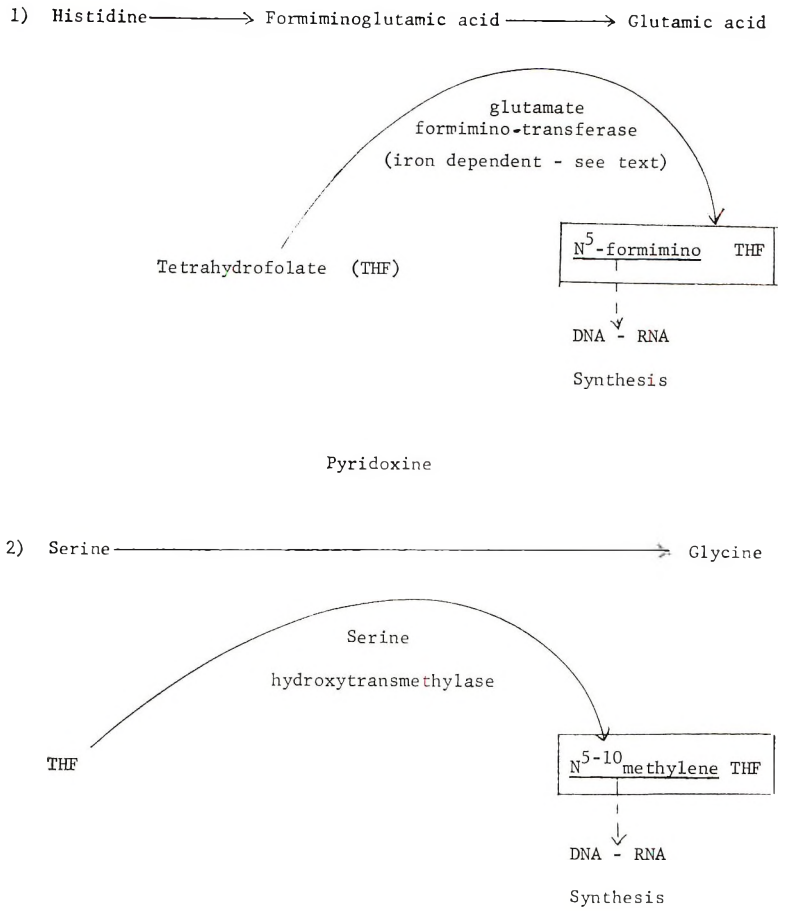


Fig. 7 Schematic illustration of one-carbon transfer to tetrahydrofolic acid.

can be induced in the rat by a deficiency of dietary iron. This defect in folate metabolism is presumably mediated through a decrease in the activity of the enzyme glutamate formimino-transferase which is dependent on iron for optimal activity.

ACKNOWLEDGMENT

We wish to thank Dr. Richard R. Streiff for his interest and his assistance in the preparation of the photomicrograph.

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# Growth Factors for *Lactobacillus bulgaricus* GS [1]

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*ABSTRACT* The study of the nutrition of lactic acid bacteria has led to the discovery or isolation of several new vitamins and growth factors of general nutritional and biochemical interest. The observation that a new strain of *Lactobacillus bulgaricus* required 2 unidentified growth factors therefore appeared worthy of further research which has yielded the following results: 1) Unidentified growth factors for *Lactobacillus bulgaricus* GS are widely distributed in natural materials from animal tissues to bacterial cells. 2) Two unidentified growth factors are required by *L. bulgaricus* GS. The factors were separated by hot, aqueous extraction of liver, yeast, or bacterial cells and precipitation at pH 9. One factor, designated precipitate factor (PF), is precipitated at pH 9; and the second factor, filtrate factor (FF), is soluble at pH 9. The PF has been purified 170-fold by precipitation at selected pH and extraction of dried precipitates with hot absolute ethanol in the presence of acid. The PF is an essential growth factor; the FF is not essential but increases the growth response of the organism to PF 3- to 6-fold. 3) PF activity from liver extract was separated by paper chromatography with 35% ethanol into 3 forms with $R_F$ values 0, 0.7 and 0.8. Two forms were present in yeast and bacterial extracts. The dominant form of PF with an $R_F$ of 0.8 exhibited a blue fluorescence and the fluorescent emission spectrum showed a peak at 433 mμ with excitation at 355 mμ. The ultraviolet absorption maximum and minimum were 266 and 245 mμ, respectively. 4) PF is soluble in acidic, absolute ethanol and slightly soluble in chloroform. It is also soluble in water at pH 2, completely insoluble at pH 9, and diffusible through cellophane. 5) Harman and tetrahydroharman can replace PF activity in tube assays but are not identical with PF. Other indole derivatives, including 3-carbolines, produced little or no growth and some inhibited growth.

Unidentified growth factors for lactic acid bacteria are of biochemical and nutritional importance because these organisms have nutritional requirements remarkably similar to higher animals. Several B-complex vitamins and many amino acids are required by both animals and lactic acid bacteria (1).

The existence of an unidentified growth factor, Georgia bulgaricus factor (GBF), for *Lactobacillus bulgaricus* Georgia Strain (GS) was demonstrated and described as a single substance. Attempts to extract and concentrate this factor from liver extract met with little success. Individual compounds and various combinations of known compounds failed to produce growth (2).

Previously, initial purification by adsorption on charcoal and elution with butanol gave a fraction with specific activity of 2.4 units/mg and a yield of 3.2% (2). Further purification of the growth factors and properties of these factors are described.

The present paper reports evidence that the organism requires 2 unidentified growth factors, one of which exists in 3 interchangeable forms. The factors are widely distributed in natural materials from animal tissues to bacterial cells. Two known indole alkaloids replaced one of them, but these alkaloids are not chemically identical with the growth factor as it occurs in the best concentrates from bacterial cells.

## MATERIALS AND METHODS

*Inoculum.* *L. bulgaricus* GS was cultured and the inoculum prepared as described by Weinman et al. (2), except that the inoculum was diluted 100-fold before addition to the final basal medium, and 2 additional vitamins, pyridoxamine phosphate (3.7 μg) and pyridoxal phosphate (0.5 μg), were added per 1 ml of final basal medium.

*Bioautography medium.* For bioautography, single-strength basal medium (100

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[2] H. M. Yang, in previous publications.

ml) was supplemented with ascorbic acid (50 mg), liver extract filtrate (15 ml, described later) and agar (1 g). This medium was autoclaved at 120° for 6 minutes and cooled to 45°, undiluted and washed inoculum (1.5 ml) added, and after thorough mixing, the medium was poured into a flat glass dish, 17 × 28 cm. Paper strips were applied to the agar surface for 1 hour, then the plates were incubated for 15 to 20 hours at 37°.

*Test tube experiments.* For experiments in test tubes, samples and standard liver extract were pipetted into triplicate sets of 1.3-cm diameter × 10-cm long Pyrex tubes, 0.3 ml of liver extract filtrate added to each tube, the volume was adjusted to 1.5 ml and the tubes were covered with aluminum caps. Both the tubes and double-strength medium were autoclaved at 120° for 6 minutes. Double-strength uninoculated basal medium (1.5 ml) was added aseptically to one set of tubes. The remaining 2 sets of tubes were filled with 1.5 ml of inoculated basal medium, and then both control and inoculated tubes were incubated at 37°. After an incubation of 18 to 25 hours, growth was measured turbidimetrically with the Spectronic 20 spectrometer at 650 mμ. Growth was expressed directly as percentage transmission or in dry weight of cells as determined from a previously prepared graph.

*Paper chromatography.* Ascending chromatograms were prepared using Whatman no. 3 MM paper cylinders developed with ethanol-water (7:13, v/v).

*Fluorescence emission spectra.* These were obtained in 0.01 N HCl using an Aminco-Keirs spectrophosphorimeter.

*Preparation of extracts from animal tissue and bacterial cells.* Standard liver extract was prepared as published (2), except that the time of autoclaving was shortened to 30 minutes.

The air-dried Torula yeast [3] was mixed with 6 volumes of water until suspended and was autoclaved at 120° for 30 minutes. The supernatant fraction was stored at − 7° after centrifuging at 4° for 10 minutes. The solids of the extract represented 46 mg/ml.

After autoclaving, vitamin $B_{12}$ fermentation cells [4] were centrifuged and the supernatant fraction was stored at − 7°.

*Standard growth curve.* A standard preparation of liver extract, stored frozen, was used throughout this study. It contained 33 mg/ml of solids. A unit of activity is defined as the amount of precipitate factor (PF, described later) in 1 mg of standard liver extract assayed with liver extract filtrate in the medium.[5]

## RESULTS

*Existence of two growth factors.* In attempts to adsorb the growth-promoting activity on charcoal, it was noted that increasing amounts were removed from solution as the pH was increased. The charcoal filtrate obtained at pH 9 was devoid of growth-promoting activity. It was found that a precipitation of growth factor activity was taking place and the charcoal (step 2 of diagram) was merely serving

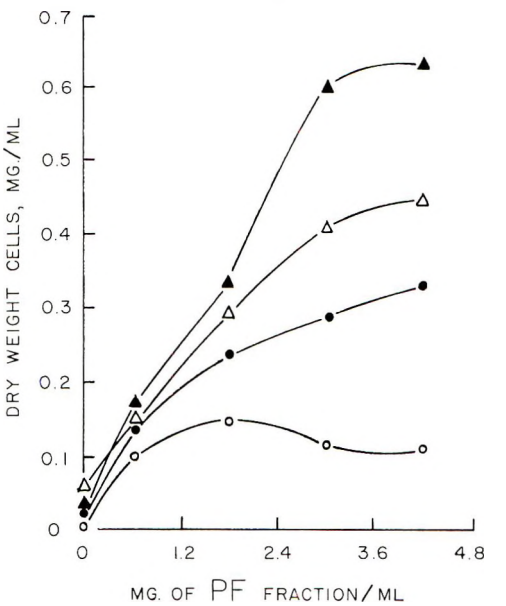


Fig. 1 Factorial experiment — the existence of 2 factors, precipitate factor (PF) and filtrate factor (FF), in liver extract. ○, no liver extract filtrate; ●, 0.03 ml filtrate; △, 0.1 ml filtrate; ▲, 0.23 ml filtrate/ml of final medium.

[3] Torula yeast was obtained from Lake States Yeast Company, Rhinelander, Wisconsin.

[4] It is a pleasure to express our appreciation to Dr. J. D. Sudarsky, Bioferm Company, Wasco, California, for vitamin $B_{12}$ fermentation cell cream and help with large scale extractions. This is a propionic acid-type fermentation for commercial production of vitamin $B_{12}$ using a crude medium.

[5] Yang, H. M., and W. L. Williams 1963 Unidentified growth factors for *Lactobacillus bulgaricus* GS. Abstracts of Amer. Soc. Microbiol., South Eastern Branch Meetings, Jacksonville, Florida.

as a filter aid. As calculated from the lower curve shown in figure 1, the precipitate redissolved at pH 2 had an apparent content of 25 units/ml in the absence of filtrate. Inclusion of 0.03, 0.1 and 0.23 ml of filtrate/ml of final basal medium markedly increased the potency of the precipitate from 25 to 97, 118 and 145 units/ml, respectively. Filtrate and precipitate obtained from vitamin $B_{12}$ fermentation bacterial cells showed a similar phenomenon. The precipitate fraction from such cells assayed 23 units/ml without filtrate and 82 units/ml with 0.1 ml filtrate/ml of final basal medium. Two such fractions were also obtained from Torula yeast extract. Thus it appeared that the organism required 2 separate unidentified growth factors rather than one, previously named GBF (2). For convenience the 2 factors were designated precipitate factor (PF) and filtrate factor (FF); FF preparations alone failed to support growth but augmented the growth response to PF up to sixfold.[6] In all subsequent experiments, a specially prepared batch of liver extract filtrate was included in the basal medium in an amount (1:10, v/v) which gave the best response curve to PF preparations.

Another line of evidence for 2 growth factors was obtained by observing growth on solid medium. Using solid medium containing just enough PF (5 units/ml) to give faint growth over the entire agar medium, a markedly different growth response to PF and FF preparations was obtained. A paper disc containing 14 PF units is shown on the left side of figure 2. This disc gave localized dense growth. On the right side of figure 2, a paper disc which contained 0.1 ml of a FF preparation gave diffuse and less dense growth. Uninoculated control agar plates were completely clear indicating absence of precipitate artifacts. It was apparent that PF diffuses more slowly than FF or was trans-

[6] See footnote 5.

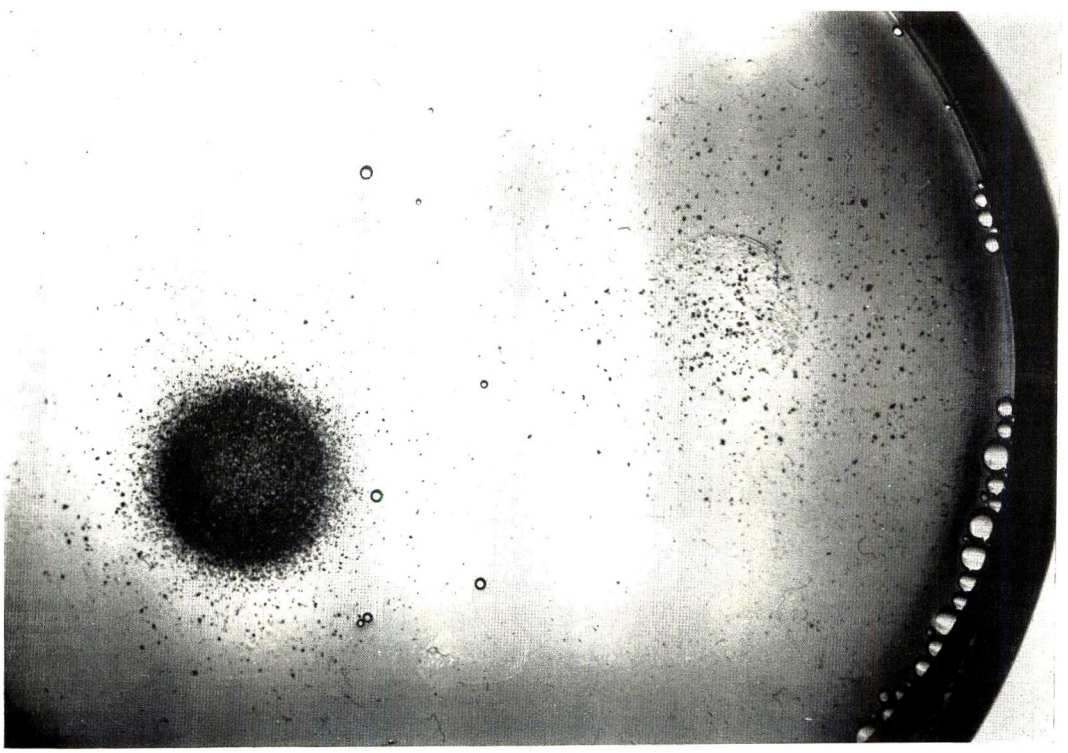

Fig. 2 The response of *Lactobacillus bulgaricus* GS to FF (right side) from liver extract and PF (left side) from extracts of vitamin $B_{12}$ fermentation cells.

ported into cells of the organism more rapidly.

*Distribution of growth factors in natural materials.* Additions of liver extract to the medium promoted growth of the organism after 17 to 25 hours incubation as shown in figure 3. Growth obtained with crude extracts or known compounds was compared with this standard growth curve in each experiment to determine potency. A variety of natural products assayed for the PF activity are shown in table 1. Hot aqueous extract of vitamin $B_{12}$ fermentation bacterial cells was the best source.

*Fractionation procedure.* An earlier report suggested that the growth factor activity was adsorbed on charcoal at an alkaline pH (2),[7] but more recent experiments show that PF was actually precipitated to the extent of 87 to 100% at pH 9.[8,9] As a result of many attempts to purify PF, the fractionation scheme shown in figure 4 was evolved. This series of steps was suitable for purification of PF from liver extract and Torula yeast extract. With the observation that PF activity precipitated in an alkaline solution and was soluble in acid solution, precipitation at an intermediate pH was investigated with the results shown in table 2. It was apparent that precipitation between pH 5 and 8 would give a good yield and improved potency and this was selected as the next purification step as indicated in figure 4, step 4.

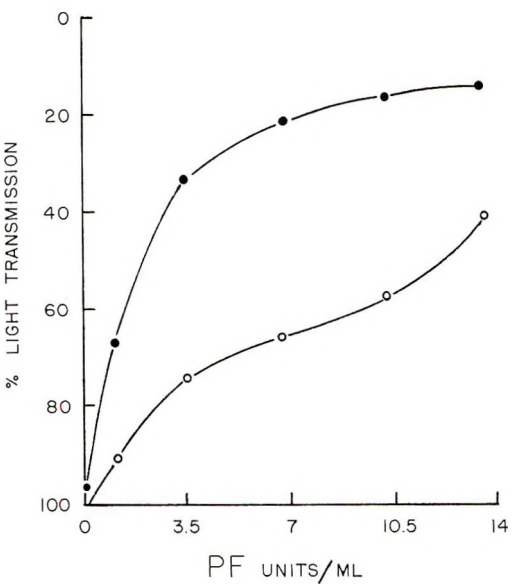


Fig. 3 The growth response of *Lactobacillus bulgaricus* GS to standard liver extract in the presence of an FF preparation. Incubation time = ○, 17 hours; ●, 25 hours.

TABLE 1

*Distribution of growth factors in natural materials*

| Substance (aqueous extract) | *units/ml* | *units/mg solids* |
|---|---|---|
| Beef liver | 33 | 1 |
| *Saccharomyces cerevisiae* | 41 | 0.7 |
| Torula yeast (air-dried) | 120 | 2.8 |
| Vitamin $B_{12}$ fermentation, liquor | 36 | 1.5 |
| Vitamin $B_{12}$ fermentation, cells | 38–313 | 2.2–8.3 |
| *Bacillus thuringiensis*, liquor | 26 | 6.0 |
| *B. thuringiensis*, cells | 110 | 3.1 |
| Glutamic acid fermentation, cells | 18 | 0.1 |

TABLE 2

*Effect of increasing pH on the solubility of precipitate factor*

| Precipitate | Potency | Yield |
|---|---|---|
| *pH range* | *units/mg* | % |
| 2–5 | 11 | 10 |
| 5–6 | 23 | 30 |
| 6–7 | 25 | 25 |
| 7–8 | 21 | 12 |
| 8–9 | 13 | 3 |

In step 5 the total yield of PF activity increased nearly threefold. This will be discussed later.

*Bioautography of precipitate factor.* Chromatography of a PF concentrate of 15 to 20 units/mg potency on Whatman no. 3 MM filter paper with a variety of solvents including butanol, ammonia-butanol, ethanol, ammonia-ethanol and ammonia-water at pH 10 in various proportions showed only one active zone which

[7] See footnote 5.

[8] Yang, H. M., G. K. Morris and W. L. Williams 1964 Two unidentified growth factors for *Lactobacillus bulgaricus* GS. Federation Proc., 23: 244 (abstract).

[9] Yang, H. M., and W. L. Williams 1964 Purification of a growth factor for *Lactobacillus bulgaricus*. Abstracts of Amer. Soc. Microbiol., South Eastern Branch Meetings, Atlanta, Georgia.

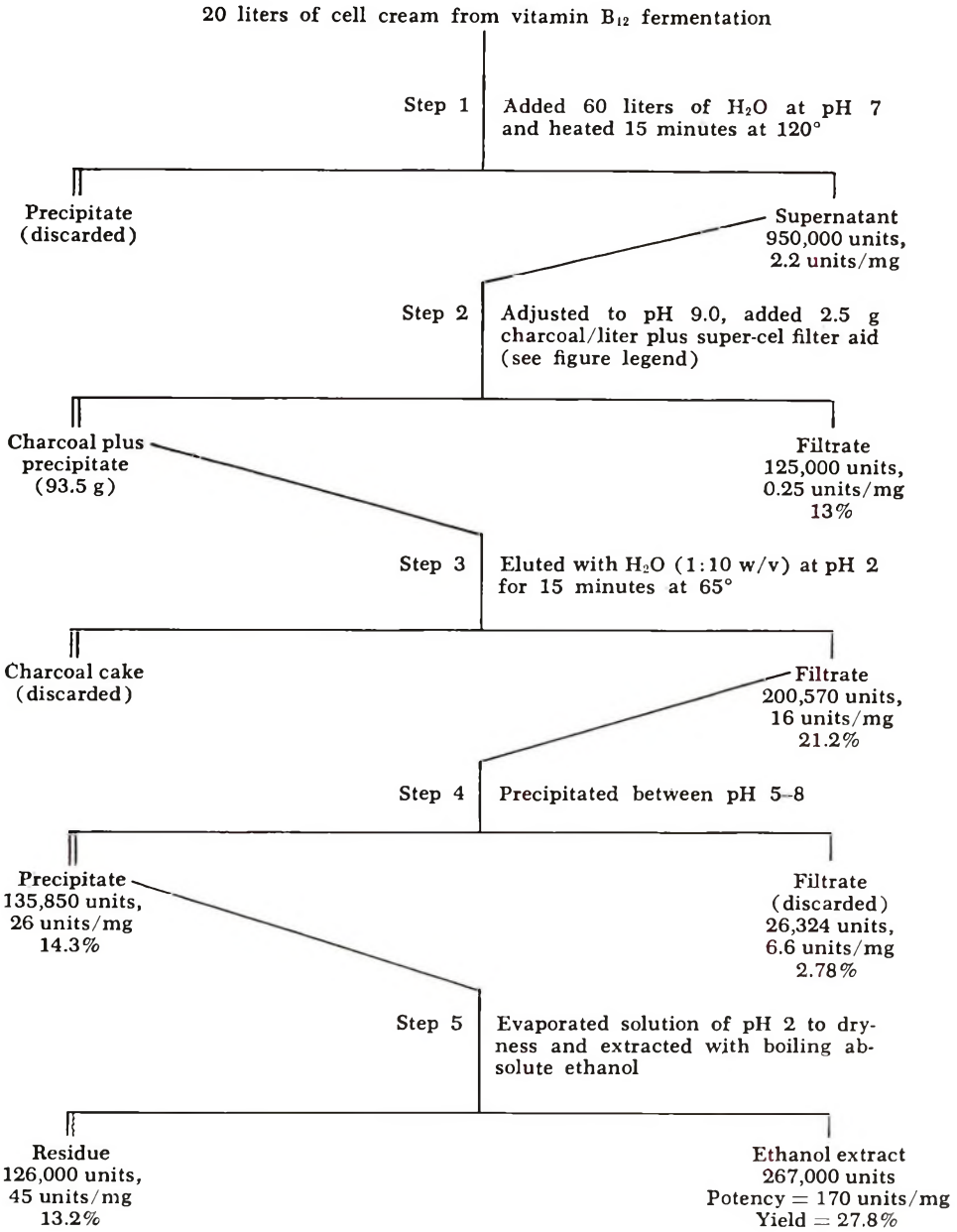


Fig. 4 Diagram of procedure for purification of growth factors. Omission of charcoal in step 2 gave an active precipitate at pH 9 and an inactive filtrate (Yang, H. M. 1964 Unidentified growth factors for Lactobacilli, Master's Thesis, University of Georgia).

stayed at the origin with the organic solvents and moved immediately behind the solvent front with the aqueous solvents. Use of ethanol-water (7:13, v/v) with PF concentrates revealed 3 different growth zones. The fastest moving form of PF was the dominant form present in liver, Torula yeast, and extracts of vitamin $B_{12}$ fermentation cells (table 3). The 3 forms have been designated PF-1 ($R_F = 0$), PF-2 ($R_F = 0.7$) and PF-3 ($R_F = 0.8$) numbering from the origin.

TABLE 3

*$R_F$ values of precipitate factor (PF) from different sources*

| No. of PF | Liver extract | Yeast extract | Vitamin $B_{12}$ fermentation extract |
|---|---|---|---|
| PF–1 | 0 | 0 | nr [1] |
| PF–2 | 0.7 | nr [1] | 0.7 |
| PF–3 | 0.8 | 0.8 | 0.8 |

[1] No growth response.

Torula yeast extract concentrate contained only PF-1 and PF-3. The PF-3 area from liver, Torula yeast and vitamin $B_{12}$ fermentation cell extracts exhibited a blue fluorescence. The PF-2 area from liver and vitamin $B_{12}$ fermentation cell extracts exhibited a yellow fluorescence.

*Properties of Precipitate Factor.* PF activity from all 3 sources was stable at 120° for 30 minutes at pH 2 to 7, but was destroyed at pH 1 at 120° for 30 minutes. PF activity was readily dialyzed through cellophane. Fractions obtained in step 3, figure 4, had lost ninhydrin-reactive materials. PF activity was soluble in hot absolute ethanol and slightly soluble in chloroform and ether. Solubility in water and dependence on pH is shown in table 2.

Since PF-3 was the dominant form in the hot aqueous extracts from each of the 3 sources, it was selected for further study. The blue fluorescent band of PF-3 obtained from the bacterial cells was cut from paper chromatograms and eluted with water. The ultraviolet-absorption maximum and minimum were at 266 and 245 mμ, respectively. The fluorescent emission spectrum showed a peak at 433 mμ with excitation at 355 mμ, suggesting a relationship to 3-carbolines.

*PF activity of known compounds.* Many known substances (2) [10,11] produced little or no growth response. The 3-carbolines, the plant alkaloids harman and tetrahydroharman, were investigated. These 2 compounds were as potent as the best PF preparation (170 to 200 units/mg) obtained in step 5, figure 4. Bioautographic studies indicated that both growth zones of harman and tetrahydroharman migrated in 35% ethanol near the PF-3 area. These compounds are not chemically identical with PF because of differences in ultraviolet-absorption spectra (fig. 5) and fluorescent emission spectra (table 4). Other indole derivatives, including carbolines, were tested on solid medium as shown in table 5. The growth observed with the indicated indole derivatives was lower compared with that of tetrahydroharman by an order of magnitude.

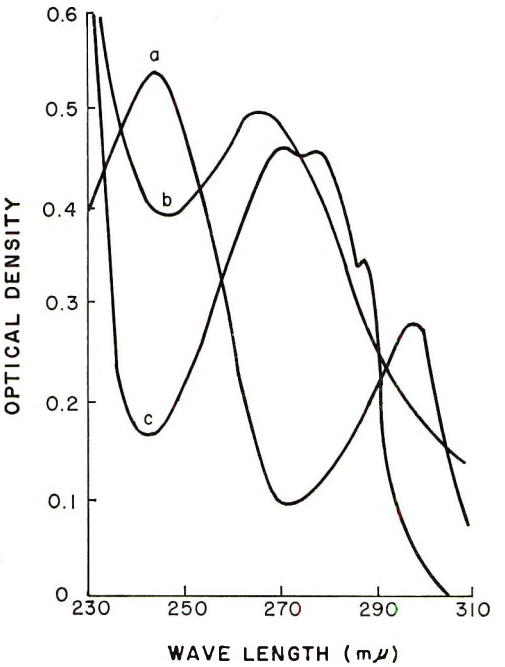


Fig. 5 Absorption spectra of (a) harman (b) PF-3 (c) tetrahydroharman in 0.01 N HCl.

TABLE 4

*Fluorescent emission spectrum*

| Substance | Emission | Excitation |
|---|---|---|
| | mμ | mμ |
| PF–3 | 433 | 355 |
| Harman | 433 | 255, 300, 370 |
| Tetrahydroharman | 350 | 295 |

## DISCUSSION

After an extensive program of testing many known compounds of nutritional or biocatalytical activity for identity with PF, it was unexpected to find that 2 indole alkaloids from plants would replace the

[10] See footnote 9.

[11] Williams, W. L., and H. M. Yang 1965 Growth factors for a lactic acid bacterium. Federation Proc., 24: 625 (abstract).

growth factor. It is tempting to speculate that these pharmacologically active plant alkaloids have a similar function in plants and *L. bulgaricus*. It is also quite possible that the growth-promoting property of these alkaloids is a biochemical coincidence.

The PF and FF appear to be chemically and metabolically unrelated growth factors. The fact that PF is essential for growth whereas small amounts of growth can be obtained without FF may mean that PF preparations contain small amounts of FF. Alternately, the organism may possess marginal ability to synthesize FF. Relatively few known compounds have been tested for replacement of FF; therefore, FF may be a known substance or combination of substances. In contrast with the separate nature of PF and FF, PF-1, PF-2 and PF-3 appear to be chemically and functionally related. The organism will grow on any one of these 3 forms and hence the 3 forms are interchangeable for growth.

An increase in yield of PF units by the treatment with acidic alcohol is shown in step 5, figure 4. Although it is possible that this step removed a growth inhibitor, a chemical change of PF to a more potent form must also be considered. The $R_f$ value of PF was unchanged by the acidic-alcohol treatment.

The possibility that PF is chemically identical to either harman or tetrahydroharman is of crucial importance. The widely differing absorption spectra of PF-3 and harman eliminates harman as being identical with PF-3. The absorption spectrum of tetrahydroharman parallels that of PF-3 somewhat more closely; however the fluorescent emission spectrum of tetrahydroharman differs markedly from that of PF-3.

The growth observed from the indole derivatives listed in table 5 was considerably less than that obtained from tetrahydroharman, indicating a more distant relationship to PF.

The discovery of new growth factors for lactic acid bacteria possesses a high potential for being of general biological significance. Folic acid, leucovorin (citrovorum

TABLE 5

*Effect of indole derivatives on growth of Lactobacillus bulgaricus GS*

| Substances [1] | Response [2] |
|---|---|
| Acetamide, N-[2-(3-indolyl)ethyl]- | G |
| 3-(2-Acetamido-ethyl)-indole | NR |
| 3-Acetylindole | NR |
| 5-(Benzyloxyl)-3-indole acetic acid | I |
| 5-(Benzyloxyl)-indole-3-carboxaldehyde | NR |
| 5-Chloroindole-2-carboxylic | I |
| Glycyl-L-tryptophan | NR |
| Glycyl-DL-tryptophan | NR |
| 5-Hydroxy-3-indole-acetic acid | G |
| 6-Hydroxy-tetrahydroharman | G |
| Indole | I |
| 3-Indole acetic acid | G |
| Indole-5-carboxylic acid | G |
| 3-Indole carboxylic acid | G |
| 3-Indole carboxaldehyde | G |
| 3-Indole ethanol | NR |
| Indoxyl acetate | I |
| Indoxyl sulfate K salt (indican) | G |
| 3-(2-Dimethylamino-ethyl)-5-hydroxyindole or butotenine | I |
| 3-(Dimethylaminomethyl) indole or gramine | NR |
| α-Methylaminomethyl-1-methyl-3-indole-methanol | G |
| 5-Methyl indole | I |
| 5-Methyoxy indole | I |
| 3,3′-Methylene bis-(2-methyl indole) | NR |
| Serotonin creatinine sulfate | G |
| Tryptamine·HCl | G |
| DL-Tryptophan | G |
| Harmalol·HCl | NR |
| 1H-Pyrido (3,4-b) indole-1,3,4 (2H, 9H)-trione, 2, 9 dimethyl- | NR |
| 1H-Pyrido (3,4-b) indole-7-ol, 3-ethyl-2,3,4,9-tetrahydro-, HCl | G |
| 1H-Pyrido (3,4-b) indole-2,3,4,9-tetrahydro-6-methoxy-1-methyl-, HCl or adrenoglomerulotropin | IG |
| 1H-Pyrido (3,4-b) indole-2,3,4,9-tetrahydro-2-methyl- | IG |
| 3H-Pyrido (3,4-b) indole-4,9-dihydro-6-methoxy-1-methyl-, HCl | IG |
| 9H-Pyrido (3,4-b) indole-6-methoxy-1-methyl or harmine | I |
| 9H-Pyrido (3,4-b) indole-6-methoxy-1,9-dimethyl-, HCl | IG |
| 9H-Pyrido (3,4-b) indole-7-methoxy-1-methyl- | I |
| N-(py)-Propyltetrahydroharman-, HCl | I |
| N-(py)-Propyltetrahydroharmine-, HCl | G |
| Tetrahydroharman carboxylic acid | G |
| Yohymbine·HCl | IG |

[1] Substances were placed directly on solid medium.

[2] G = Growth response; I = inhibition; NR = no growth response; IG = inner zone of inhibition with outer zone of growth.

factor), lipoic acid, and in fact, over half of the B vitamins were first known as growth factors for yeasts or bacteria (1). The growth factor would be of general interest and importance if it proved to be no more than a new indole derivative in view of the known wide physiological activity of indole compounds.

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# Effect of Calcium, Phosphorus and Zinc on Zinc-65 Absorption and Turnover in Rats Fed Semipurified Diets [1]

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*ABSTRACT* The objectives of this study were to assess the effects of dietary levels of calcium, phosphorus and zinc on zinc absorption and turnover in rats fed semipurified diets. In 7 experiments $^{65}$Zn-glycine was administered by diet, gavage and intramuscular injection to growing rats fed the various diets and $^{65}$Zn absorption and turnover were assessed by techniques described previously. Calcium decreased ($P < 0.01$) absorption of $^{65}$Zn in the presence of approximately 1% dietary inorganic phosphorus but not when phosphorus was 0.3 to 0.5%. Intermediate levels of phosphorus gave variable results. Calcium decreased ($P < 0.05$) the biological half-life of oral $^{65}$Zn zero to 44 hours post-administration and increased ($P < 0.01$) it 100 to 300 hours post-administration, both effects being independent of dietary phosphorus. Calcium decreased ($P < 0.01$) the proportion of the body $^{65}$Zn in liver and kidneys at 345 hours post-administration, and increased ($P < 0.05$) the proportion in bone. When the diet contained sufficient inorganic phosphorus, the effects of calcium on $^{65}$Zn metabolism with a semipurified diet containing protein of animal origin and no phytic acid were analogous to those observed earlier in rats fed a practical diet containing plant protein. Omitting zinc from a diet containing casein hydrolysate permitted almost complete absorption of dietary $^{65}$Zn and markedly increased body retention of $^{65}$Zn. Zinc deficiency caused a greater proportion of the body $^{65}$Zn to be in the liver at 385 hours post-administration and a smaller proportion to be in bone.

Numerous dietary variables have been reported to affect zinc absorption and metabolism. An earlier report from this laboratory (1) showed that increased dietary calcium decreased the absorption of $^{65}$Zn and altered its biological half-life and distribution in rats fed a practical diet. Addition of calcium, with or without phosphate, to practical swine diets greatly aggravates the zinc deficiency syndrome, whereas addition of phosphate alone helps alleviate the dermatitis associated with zinc deficiency in swine but has little effect on growth (see (2) for references). However, Cabell and Earle (3) reported that calcium and inorganic phosphorus independently caused a conditioned zinc deficiency in rats fed a semipurified diet containing soybean protein and that the effect was additive. Other workers have fed semipurified diets to rats (4) or chicks (5–7) and observed decreased zinc absorption or availability due to calcium, but only in the presence of added phytic acid. Supplementary zinc has been shown to decrease $^{65}$Zn uptake and retention in rats fed a practical diet (8) and chicks fed a low zinc, semipurified diet (9), but $^{65}$Zn absorption data were not reported in either case.

The 7 experiments reported here represent a continuation of studies of dietary factors affecting $^{65}$Zn absorption and retention in rats. However semipurified, rather than practical, diets were fed. The effects of dietary calcium and phosphorus (experiments 1–5) and of dietary zinc (experiments 6–7) were investigated.

## METHODS AND MATERIALS

Male weanling rats of the Holtzman strain were used in all experiments. The animals were housed in galvanized wire cages in experiments 1–5 and stainless steel or Monel metal cages in experiments 6 and 7. Distilled water in glass water bottles and feed in porcelain feed cups were provided ad libitum. Samples of the basal ration (table 1) in each of the first

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TABLE 1

*Basal diets*

| | Exps. 1–5 [1] | Exps. 6–7 |
|---|---|---|
| | *g/kg* | *g/kg* |
| Protein source | 182.00 [2] | 182.20 [3] |
| Glucose monohydrate [4] | 678.31 | 712.70 |
| Cellulose [5] | 50.00 | — |
| Corn oil | 50.00 | 50.00 |
| Mineral mixture | 28.80 [6] | 46.20 [7] |
| Vitamin mixture | 10.86 [8] | 8.90 [9] |
| Zinc supplements | 0.029 [10] | — [11] |

[1] Calcium and phosphorus were varied by the addition of $CaCO_3$ and $KH_2PO_4$, respectively, at the expense of glucose monohydrate.

[2] Alcohol-extracted casein, laboratory prepared from crude casein; or isolated soy protein, Archer-Daniels-Midland Company, Cincinnati.

[3] Salt-free, acid hydrolyzed casein plus 2.2 g tryptophan, General Biochemicals, Chagrin Falls, Ohio.

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

[5] Solka-floc, Brown Company, New York.

[6] Provided in g/kg of diet: $CaCO_3$, 4.8; $KH_2PO_4$, 8.4; NaCl, 12.5; $MgSO_4$, 2.43; ferric citrate, 0.31; $CuSO_4$, 0.06; $MnSO_4 \cdot H_2O$, 0.06; KI, 0.00025; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.000175. In addition, 0.1 g $MnSO_4$ and 4.95 g $MgSO_4$ were provided/kg in experiments 4 and 5.

[7] Same as footnote 6 except: $CaCO_3$, 12.1; $CaHPO_4$, 0.27; $KH_2PO_4$, 17.06.

[8] Provided in mg/kg of diet: thiamine·HCl, 5; riboflavin, 5; niacin, 25; Ca pantothenate, 20; pyridoxine·HCl, 2.5; folic acid, 0.2; menadione, 0.5; biotin, 0.1; vitamin $B_{12}$, 0.02; ascorbic acid, 50; inositol, 100; choline chloride, 1760 and (in IU) vitamin A, 4000; vitamin $D_2$, 8000; and vitamin E, 100.

[9] Same as footnote 8 except: choline chloride, 1400; vitamin A, 5000.

[10] Supplement added as zinc carbonate. Complete, air-dried diets analyzed 28, 22, 22, 17, and 27 ppm zinc for experiments 1 through 5, respectively.

[11] Basal diets analyzed 2.5 ppm zinc and diets supplemented with zinc oxide analyzed 20 ppm.

5 experiments were analyzed after dry ashing for calcium and zinc by atomic absorption spectrophotometry. The rations contained approximately 0.20% calcium (air-dry basis) except in experiment 1 where it was decreased to 0.11% by deletion of a portion of the calcium from the mineral mix. The supplementary calcium carbonate analyzed less than 5 ppm zinc. Total phosphorus analyses of the rations were by a modification of the method of Fiske and Subbarow (10) following wet oxidation. Except for zinc determination, chemical analyses were not made in experiments 6 and 7, but by calculation these rations contained 0.5% calcium and 0.4% phosphorus.

Each treatment group receiving $^{65}$Zn-glycine complex in the feed or by gavage in experiments 2 through 5 had 8 rats. In experiment 1 and in all treatment groups receiving $^{65}$Zn-glycine intramuscularly, 4 rats were used per treatment. Injected animals in each treatment group are necessary in the procedure used to calculate $^{65}$Zn absorption (1). Fewer rats per treatment group were used in experiments 6 and 7. Differences between treatment means were analyzed for statistical significance by applying the analysis of variance described by Steel and Torrie (11).

Preadministration treatment of the rats, method of preparation of the $^{65}$Zn-glycine complex, isotope administration and whole-animal $^{65}$Zn assay procedures, and method of calculating $^{65}$Zn absorption and biological half-life were as described previously (1).

## RESULTS AND DISCUSSION

*Effects of calcium and phosphorus on growth.* For simpler interpretation of data on $^{65}$Zn absorption and turnover it was desired that the dietary variables, calcium and phosphorus, not affect growth rate. Rats fed the basal level of calcium (0.11%) in experiment 1 showed a marked decrease in growth compared with those that received supplemental calcium (table 2). Increasing calcium in the basal diet to 0.20% (exp. 2) resulted in animals growing at rates identical to those receiving 0.80% calcium (basal + 0.60% Ca), but those receiving the basal plus 1.76% additional calcium showed decreased growth. In previous studies (1) practical diets containing approximately the same levels of calcium, but with somewhat higher levels of phosphorus, were fed to rats with no significant difference in growth due to calcium. The pronounced differences in growth between treatments were eliminated when the basal diet contained 0.20% calcium and the levels of calcium supplementation were + 0.50% and + 1.25% (exps. 3–5). Due to rancid corn oil, growth was inadvertently restricted in some treatment groups during the early part of the pre-experimental period in experiment 3 (i.e., table 2, experiments 3A and 3C); however, feeding of fresh diets before the experimental period started resulted in all animals growing at comparable rates during the experimental period.

*Effects of calcium and phosphorus on $^{65}$Zn absorption.* Calcium sometimes depressed $^{65}$Zn absorption from the semipurified diets (table 3), although less consistently than had been previously observed with practical diets (1). The ability of calcium to

TABLE 2

*Effect of dietary calcium and phosphorus on body weight*

| Exp. | P in basal diet | Days fed experimental diet | Final [1] body weight | | |
|---|---|---|---|---|---|
| | | | Basal | + 0.50% Ca [2] | + 1.25% Ca [3] |
| | % | | g | g | g |
| 1 | 0.48 | 47 | 150 A [4] | 269 C [4] | 249 B [4] |
| 2 | 0.52 | 49 | 268 A | 269 A | 207 B |
| 3A | 0.61 | 41 | 204 A | 236 C | 218 B |
| 3B [5] | 0.78 | | 258 A | 258 A | 249 A |
| 3C [6] | 0.62 | | 214 A | 211 A | 220 A |
| 4 | 0.31 | 34 | 213 A | 210 A | 194 B |
| | 0.66 | | 197 A | 216 B | 212 B |
| | 0.96 | | 197 A | 207 A | 208 A |
| 5 | 0.44 | 31 | 185 A | 182 A | 173 A |
| | 1.09 | | 182 A | 189 A | 190 A |

[1] Initial body weights were similar between treatments. The basal diets contained 0.11% Ca in experiment 1 and 0.20% Ca in all other experiments.
[2] Changed to + 0.60% Ca in experiments 1 and 2.
[3] Changed to + 1.76% Ca in experiments 1 and 2.
[4] Treatment means. Values not followed by same letter within the same line are significantly different ($P < 0.05$).
[5] Diet supplemented with 0.32% phytic acid (General Biochemicals, Chagrin Falls, Ohio) which provided 0.17% additional phosphorus. The phytic acid and total phosphorus values were by laboratory analysis.
[6] Diet contained soy protein. All other diets in experiments 1–5 employed casein.

TABLE 3

*Effect of calcium and phosphorus on absorption of $^{65}$Zn* [1]

| Exp. | P in basal diet | Method of $^{65}$Zn administration | Administered $^{65}$Zn absorbed | | | Significance due to calcium |
|---|---|---|---|---|---|---|
| | | | Basal | + 0.50% Ca [2] | + 1.25% Ca [3] | |
| | % | | % | % | % | |
| 1 | 0.48 | diet | | 30.6 A [4] | 21.3 A [4] | > 0.1 |
| | | gavage | | 28.6 A | 29.8 A | > 0.1 |
| 2 | 0.52 | diet | 34.2 A | 35.1 A | | > 0.1 |
| | | gavage | 27.4 A | 30.9 A | | > 0.1 |
| 3A | 0.61 | diet | 32.3 A | 21.8 B | 19.5 B | < 0.01 |
| | | gavage | 31.5 A | 30.4 A | 21.4 B | < 0.05 |
| 3B [5] | 0.78 | diet | 31.6 A | 30.0 A | 27.6 A | > 0.1 |
| | | gavage | 31.7 A | 31.6 A | 29.4 A | > 0.1 |
| 3C [6] | 0.62 | diet | 18.7 A | 13.8 B | 15.6 B | < 0.05 |
| | | gavage | 17.0 A | 15.6 A | 14.4 A | > 0.1 |
| 4 | 0.31 | diet | 23.9 A | 25.5 A | 23.4 A | > 0.1 |
| | 0.66 | diet | 31.7 A | 30.3 A | 25.8 A | > 0.1 |
| | 0.96 | diet | 29.6 A | 31.3 A | 23.2 B | < 0.01 |
| 5 | 0.44 | diet | 22.1 A | 21.9 A | 18.5 A | > 0.1 |
| | 1.09 | diet | 26.4 A | 19.3 B | 17.0 B | < 0.01 |

[1] Data for treatment groups in which there was a pronounced effect of calcium of body weight are not included. Method described in reference (1).
[2] See footnote 2, table 2.
[3] See footnote 3, table 2.
[4] See footnote 4, table 2.
[5] See footnote 5, table 2.
[6] See footnote 6, table 2.

decrease the absorption of orally administered $^{65}$Zn from a semipurified diet depended upon the level of dietary phosphorus; calcium decreased the absorption of $^{65}$Zn only in the presence of sufficient phosphorus. This was clearly demonstrated in experiments 4 and 5 (table 3) which showed no significant effect of calcium on $^{65}$Zn absorption when dietary phosphorus was either 0.31 or 0.44%, but a highly significant calcium depression of $^{65}$Zn absorption when phosphorus was increased to 0.96 or 1.09%. Similarly, calcium did not significantly decrease $^{65}$Zn absorption in experiments 1 and 2 where dietary phosphorus was 0.48% and 0.52%. From the data in experiments 3A and 4, apparently about 0.6 to 0.7% phosphorus

in a casein-containing diet was marginal and a calcium effect on $^{65}Zn$ absorption was not always significant. These results demonstrate that with sufficient inorganic phosphorus, increased dietary calcium decreased the absorption of orally administered $^{65}Zn$ from a semipurified diet containing only animal protein. This antagonistic effect of calcium on $^{65}Zn$ absorption was more dependent upon dietary phosphorus in semipurified than in practical diets.[2] The results of experiments 4 and 5 also appear to indicate an effect of phosphate per se on zinc absorption. At the lowest level of calcium (0.2%) phosphorus levels of 0.66% or higher apparently increased zinc absorption above that observed with 0.31 and 0.44% phosphorus, and at 1.45% calcium, phosphate tended to decrease zinc absorption. However, neither effect was statistically significant and effects of phosphate at 0.7% calcium were equivocal.

Other workers have also observed decreased zinc absorption or utilization with increased calcium in semipurified diets (4–7), but have attributed it to the presence of phytic acid. Such was not the case in the present studies, since the casein-glucose monohydrate [3] diets contained no added phytic acid and were presumably free of the phytate. Furthermore, the addition of 0.32% phytic acid (exp. 3B) to a diet containing casein as a protein source did not potentiate the calcium effect. Likuski and Forbes (4) demonstrated, using conventional balance techniques, that with 2.0% phytic acid in a semipurified diet, a marked effect of calcium on zinc absorption occurred, but with 0.4% phytic acid little calcium depression of zinc absorption was noted. In a separate experiment,[4] levels of phytic acid approaching 2% caused very poor growth and death of rats for an unknown reason and $^{65}Zn$ absorption could not be assessed. The present study suggests that an important control in experiments in which the effect of phytic acid on the Ca:Zn interaction is studied is to add comparable amounts of phosphorus as inorganic phosphate. It appears that a calcium depression of zinc absorption can occur in the presence of a high level of either phytic acid or inorganic phosphate; however phytic acid is possibly the more effective of the two because of its poor absorption. The calcium depression of dietary $^{65}Zn$ absorption was also statistically significant when 0.62% phosphorus was fed and isolated soy protein replaced casein (exp. 3C). The percentage of $^{65}Zn$ absorption was consistently less from the soy protein diets than from casein diets at each level of calcium. Administration of $^{65}Zn$ by gavage gave results similar to those obtained with the same diet when the isotope was administered in the feed.

*Effects of calcium and phosphorus on $^{65}Zn$ turnover.* Effects of calcium on $^{65}Zn$ turnover analogous to those noted with a practical diet (1) were consistently observed (table 4). Although only data from

[2] Unpublished data, D. A. Heth and W. G. Hoekstra.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] See footnote 2.

TABLE 4

*Effect of dietary calcium and phosphorus on turnover of $^{65}Zn$ administered in the diet*

| Period post-administration | Exp. | P in basal diet | Biological half-life of $^{65}Zn$ | | | Significance due to calcium |
|---|---|---|---|---|---|---|
| | | | Basal | + 0.50% Ca | + 1.25% Ca | |
| *hours* | | % | *hours* | *hours* | *hours* | |
| 0–44 | 4 | 0.31 | 16.2 A [1] | 14.0 A [1] | 11.7 A [1] | > 0.1 |
| | | 0.66 | 13.8 A | 13.3 A | 10.6 A | > 0.1 |
| | | 0.96 | 20.3 A | 14.5 AB | 10.4 B | < 0.05 |
| | 5 | 0.44 | 11.3 A | 7.8 B | 7.8 B | < 0.05 |
| | | 1.09 | 10.8 A | 8.2 AB | 7.1 B | < 0.05 |
| 104–269 | 4 | 0.31 | 386 A | 497 B | 551 B | < 0.01 |
| | | 0.66 | 396 A | 568 B | 560 B | < 0.01 |
| | | 0.96 | 401 A | 548 B | 577 B | < 0.01 |
| 104–310 | 5 | 0.44 | 409 A | 598 B | 453 A | < 0.01 |
| | | 1.09 | 407 A | 582 B | 548 B | < 0.01 |

[1] See footnote 4, table 2.

experiments 4 and 5 are presented, the results are typical of those obtained in experiments 1–3. Calcium decreased the biological half-life of $^{65}$Zn administered in the diet during the period zero to 44 hours post-administration, although not all the differences were statistically significant. During this time-interval the orally administered $^{65}$Zn was passing through the intestinal tract and a substantial portion (usually about 65 to 80% of the dose) was excreted. The decreased biological half-life of the orally administered $^{65}$Zn at zero to 44 hours post-administration indicated that calcium increased the rate of $^{65}$Zn excretion. As observed previously (1), calcium had the opposite effect on body $^{65}$Zn excretion during the period 100 to 300 hours post-administration, as evidenced by the significant increase in the biological half-life on $^{65}$Zn due to calcium. This effect of calcium was statistically significant in all experiments but one (exp. 3C) and was most noticeable in comparing the low and medium levels of calcium. In previous studies with practical diets (1) the increased biological half-life of $^{65}$Zn during the period 100 to 300 hours was believed to reflect a continued decreased absorption of stable zinc with increased calcium, presumably resulting in the radioactive zinc remaining in the body tissues longer. Results of the present experiments suggest that the effect of calcium on the biological half-life of $^{65}$Zn may be, in part, independent of the absorption effect, since it was observed whether or not calcium depressed $^{65}$Zn absorption. Calcium exerted its effects on biological half-life of $^{65}$Zn independent of the level of dietary phosphorus.

*Effects of calcium on tissue distribution of $^{65}$Zn.* Body distribution of $^{65}$Zn was assessed in experiment 3. Calcium altered the distribution of $^{65}$Zn remaining in the body at 345 hours post-administration (table 5). It caused a highly significant decrease in the proportion of body $^{65}$Zn in the liver and kidneys regardless of whether the diet contained casein, casein plus supplemental phytic acid or soy protein. Conversely, calcium significantly increased the amount of the body $^{65}$Zn observed in femurs of rats fed either casein or soy protein diets, but apparently not those fed casein plus supplemental phytic acid (this observation was not verified). These results substantiate those reported previously with a practical diet (1). Although the pelt contained a substantial portion of the carcass $^{65}$Zn at killing, the amount was unaffected by calcium. The effect of calcium at prolonged times after $^{65}$Zn administra-

TABLE 5

*Effect of dietary calcium on distribution of orally administered $^{65}$Zn at 345 hours post-administration (exp. 3)*

| Organ | Treatment | Portion of carcass $^{65}$Zn/organ | | | Significance due to calcium |
|---|---|---|---|---|---|
| | | Basal | + 0.50% Ca | + 1.25% Ca | |
| | | % | % | % | |
| Liver | casein | 4.41 A [1] | 3.92 B [1] | 2.72 C [1] | < 0.01 |
| | casein + 0.32% phytic acid | 4.74 A | 4.17 B | 3.88 B | < 0.01 |
| | soy protein | 4.24 A | 3.85 B | 3.38 C | < 0.01 |
| Kidney | casein | 0.63 A | 0.50 B | 0.38 C | < 0.01 |
| | casein + 0.32% phytic acid | 0.67 A | 0.61 B | 0.54 C | < 0.01 |
| | soy protein | 0.68 A | 0.64 A | 0.52 B | < 0.01 |
| Femur | casein | 1.70 A | 1.77 AB | 1.93 B | < 0.05 |
| | casein + 0.32% phytic acid | 1.92 A | 1.79 A | 1.87 A | > 0.1 |
| | soy protein | 1.79 A | 1.93 A | 2.25 B | < 0.01 |
| Pelt | casein | 21.2 A | 21.8 A | 23.3 A | > 0.1 |
| | casein + 0.32% phytic acid | 15.9 A | 15.4 A | 16.3 A | > 0.1 |
| | soy protein | 17.4 A | 15.6 A | 15.9 A | > 0.1 |
| GI tract | casein | 7.00 A | 6.08 B | 5.02 C | < 0.01 |
| | casein + 0.32% phytic acid | 5.83 A | 5.54 A | 5.03 A | > 0.1 |
| | soy protein | 5.11 A | 5.82 B | 5.20 A | < 0.05 |

[1] See footnote 4, table 2.

tion in decreasing the amount of $^{65}Zn$ in the more readily exchangeable zinc pools such as liver and kidney and increasing the amount of $^{65}Zn$ in the slower-exchanging, skeletal zinc pool may, in part, explain the slower body $^{65}Zn$ turnover caused by calcium.

The proportion of body $^{65}Zn$ observed in the gastrointestinal tract with its contents in the casein-fed rats was significantly decreased with elevated calcium. A similar trend existed with the phytic acid-supplemented diet, but the results were less clear in the rats fed the soy protein diet. This trend toward decreased intestinal loss of body $^{65}Zn$ substantiates the decreased body turnover of $^{65}Zn$ due to calcium that is observed at extended periods post-administration.

*Effect of dietary zinc on $^{65}Zn$ absorption and turnover.* Growth of rats fed the ration lacking supplemental zinc was very significantly decreased (table 6). The rats fed the low zinc ration also showed other deficiency symptoms including dermatitis and rough hair coat. When tracer $^{65}Zn$-glycine was administered orally to the animals fed the low zinc ration, they absorbed essentially the entire dose. In contrast, those receiving the same diet but with adequate zinc absorbed about 60% to 75% of the administered dose. As expected, the zinc-deficient rats also retained $^{65}Zn$ much more effectively than did rats fed the zinc-supplemented ration (93% vs. 38–55% retained at 233 hours post-administration).

The higher zinc absorption noted for rats receiving adequate zinc in experiments 6 and 7 than for similar rats in the other experiments was shown to be due, at least in part, to 2 dietary changes, (a) replacing casein with casein hydrolysate, and (b) omission of dietary bulk (cellulose).

TABLE 6

*Effect of dietary zinc on body weight and $^{65}Zn$ absorption*

| Exp. | Dietary zinc | Mean final [1] body wt | Administered $^{65}Zn$ absorbed |
|---|---|---|---|
| | *ppm* | *g* | % |
| 6 | 2.5 | 101 | 98(2) [2] |
| | 20 | 150 [3] | 61(4) [4] |
| 7 | 2.5 | 138 | 95(4) |
| | 20 | 193 [3] | 74(5) [3] |

[1] After receiving the experimental diets for 30 and 34 days for experiments 6 and 7, respectively.
[2] Number of rats.
[3] Significantly different from zinc-deficient treatment; $P < 0.001$.
[4] Significantly different from zinc-deficient treatment; $P < 0.02$.

TABLE 7

*Effect of dietary zinc on distribution of $^{65}Zn$ at 385 hours post-administration (exp. 7)*

| Sample | Portion of carcass $^{65}Zn$ | | Significance due to zinc |
|---|---|---|---|
| | 2.5 ppm zinc | 20 ppm zinc | |
| | % | % | |
| Entire liver | 6.83 [1] | 3.32 [1] | $< 0.01$ |
| Femur + tibia | 1.51 [1] | 1.94 [1] | $> 0.1$ |

[1] Mean of 3 observations.

Zinc deficiency increased the percentage of body $^{65}Zn$ observed in liver at 385 hours post-administration (table 7) and decreased that in bone. Also, in laying chickens both dietary zinc and calcium decreased the proportion of $^{65}Zn$ in soft tissues and increased it in the bones at extended periods post-administration (12). It appears that, in this respect, calcium and zinc exert similar effects on distribution of $^{65}Zn$ in the rat, but the mechanisms probably differ. Zinc may increase bone $^{65}Zn$ because the skeleton acts as a reservoir for zinc, whereas calcium increases bone $^{65}Zn$ because zinc deposition in bone is associated with the process of calcification (13, 14).

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# Ascorbic and Dehydroascorbic Acids in Guinea Pigs and Rats [1]

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*ABSTRACT* The reduction of dehydro-L-ascorbic acid to L-ascorbic acid was studied in normal guinea pigs by means of tracer techniques. Although more than half of the orally administered dehydro-L-ascorbic acid was reduced to L-ascorbic acid only 20% was reduced after intraperitoneal injection. No appreciable conversion of diketo-L-gulonic to L-ascorbic acid was observed. These results are in agreement with previous studies involving bioassay techniques which showed that while dehydro-L-ascorbic acid has antiscorbutic activity, diketo-L-gulonic acid does not. Pretreatment with 3-methylcholanthrene, which stimulates the biosynthesis of L-ascorbic acid, caused no marked increase in the reduction of dehydro-L-ascorbic acid in rats.

Animal metabolism studies with labeled L-ascorbic acid and its metabolites have been made in this (1, 2) and other laboratories (3–7). The present investigation was carried out to expand the above studies, and in particular, experiments concerned with the reduction of dehydro-L-ascorbic acid.

## MATERIALS AND METHODS

Published methods were used to prepare L-ascorbic acid-1-$^{14}$C (8) L-ascorbic acid-6-$^{14}$C (9),[2] D-ascorbic acid-1-$^{14}$C (11),the corresponding dehydroascorbic acids and diketo-L-gulonic acid (6, 12). The specific activity of the tracers ranged about 1 μc/mg, with the exception of L-ascorbic acid-6-$^{14}$C (0.3 μc/mg).

Male, non-albino guinea pigs, weighing 250 to 350 g were used.[3] To attain a stable body pool of the vitamin in guinea pigs, the following procedure was used: prior to an experiment, they were given ad libitum both water and a vitamin C-free diet (13).[4] This regimen was supplemented with daily 5-mg doses of vitamin C dissolved in 1 ml of 1% sucrose, administered by dropper. After 7 days, the animals were given the appropriate tracer. Concurrent studies with animals from the same group showed that a body pool of 50 to 60 mg/kg (average, 54) was achieved (14, 15). Male rats of the Wistar strain [5] (275 to 340 g) were maintained with a basal diet consisting of a 1:1 mixture of evaporated milk and water (16) [6] one week prior to and during experiments. The labeled compounds were dissolved in 1 ml of water and administered either by stomach tube or by intraperitoneal injection. 3-Methylcholanthrene [7] was given dissolved in corn oil [8] (20 mg/ml).

The methods for collection and preparation of samples and their assay for $^{14}$C have been described (4). To determine the amount of conversion of dehydro-L-ascorbic and diketo-L-gulonic to L-ascorbic acid a previously reported isotope dilution technique was used (14). The procedure was as follows: 24 hours after administration of the tracer, the animal was killed and a trichloroacetic acid extract of organs high in vitamin C concentration was prepared (liver, spleen, adrenals, testes and kidneys). An aliquot was used to determine the L-ascorbic acid content by the indophenol dye titration method (17), and to another aliquot carrier L-ascorbic acid

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[2] Some of the modifications described by Frush and Isbell (10) were utilized in the last steps of the synthesis.

[3] Rockland Farms, New City, New York.

[4] Powder Diet, Nutritional Biochemicals Corporation, Cleveland. Composition: heated skim milk, 30; rolled oats, 39; wheat bran, 20; codliver oil, 2; cottonseed oil, 8%.

[5] Twin Oaks Farms, Moorestown, New Jersey.

[6] Carnation Company, New York.

[7] Eastman Kodak, Rochester, New York.

[8] Mazola, Corn Products Company, New York.

was added. The L-ascorbic acid was then isolated by an anion column exchange technique and its 2,4-dinitrophenylosazone derivative was prepared. From the osazone and the chemical analysis the specific activity of body L-ascorbic acid was determined. This value was multiplied by the accepted body pool (14) and thus conversion of the precursors of L-ascorbic acid was obtained. Among the advantages of this technique are that fewer and normal animals need be utilized to obtain quantitative data as compared with standard bioassay methods.

To determine the amounts of labeled L- and D-ascorbic acid and of metabolites in urine, a previously described anion exchange column technique was used (1). Oxalic acid was isolated from urine as in earlier studies (4). Urinary L-ascorbic was measured by the indophenol dye method (17).

To study the conversion of L-ascorbic acid-6-$^{14}$C to liver glycogen, rats were first given 6 g/kg of D-glucose orally and immediately injected intraperitoneally with 2 mg of the tracer; the animals were killed 3 hours later and glycogen was isolated by a published method (18).

## RESULTS

*Comparison of the metabolism of dehydro-L-ascorbic acid-1-$^{14}$C and L- ascorbic acid-1-$^{14}$C in guinea pigs.* Guinea pigs were administered dehydro-L-ascorbic acid-1-$^{14}$C by stomach tube; the $^{14}CO_2$ exhaled and $^{14}$C moieties excreted in urine during 24 hours were then determined (table 1). The remaining $^{14}$C was calculated by difference and ranged from 45 to 51%. Isotope dilution experiments carried out as in an earlier study (14) indicated that at least 95% of the $^{14}$C remaining in the animal at 24 hours consisted of L-ascorbic acid, regardless of whether dehydro-L-ascorbic acid-1-$^{14}$C was administered by stomach tube or by intraperitoneal injection. Since the amount of $^{14}$C in the animal at 24 hours is equivalent to the amount of L-ascorbic acid remaining, it can be used to evaluate the efficiency of the reduction of dehydro-L-ascorbic-1-$^{14}$C to vitamin C.

For comparison, the results of metabolism studies with intraperitoneally administered dehydro-L-ascorbic acid-1-$^{14}$C as well as intraperitoneally and orally administered L-ascorbic acid-1-$^{14}$C are shown in table 1. After oral administration of dehydro-L-ascorbic acid-1-$^{14}$C and L-ascorbic acid-1-$^{14}$C an average of 48 and 59%, respectively, remained. In contrast, after intraperitoneal administration the percentages were 15 and 71, respectively.

*Studies with D-ascorbic acid-1-$^{14}$C and dehydro-D-ascorbic acid-1-$^{14}$C.* It has been shown (11) that intraperitoneally administered D-ascorbic acid 1-$^{14}$C is rapidly excreted in guinea pigs and rats; more than half of the tracer was found unchanged in urine. In the present study, when D-ascorbic acid-1-$^{14}$C was administered orally to guinea pigs, 78 to 81% of the $^{14}$C was eliminated as $^{14}CO_2$. In addition, 2 rats were each given 3 mg of D-ascorbic acid-1-$^{14}$C orally and after 72 hours 46 to 48% of the $^{14}$C could be accounted for as $^{14}CO_2$ and 7 to 12% in urine, which suggests incomplete absorption.

Following intraperitoneal administration of dehydro-D-ascorbic acid-1-$^{14}$C to guinea pigs, about half the tracer was excreted as $^{14}CO_2$ and the remainder in urine (table 1). Fractionation of this urine indicated than an average of 4.8% of the urinary $^{14}$C consisted of D-ascorbic acid, demonstrating some reduction. Additional fractionation showed the presence of 20% oxalic acid, and 18% of diketo-D-gulonic plus dehydro-D-ascorbic acid; 52% was unaccounted for.

When dehydro-L-ascorbic acid-1-$^{14}$C was administered intraperitoneally to guinea pigs, 48.3 and 50.6% of the $^{14}$C was excreted in urine, results similar to those obtained with the D-isomer. Fractionation of the urine gave the following average percentages of urinary $^{14}$C: diketo-L-gulonic plus dehydro-L-ascorbic, 17%; L-ascorbic acid, 1.9%; and oxalic acid, 23%. Urine of a guinea pig that received an oral dose of dehydro-L-ascorbic acid, differed only in that the L-ascorbic acid fraction was higher (14%).

*Conversion of dehydro-L-ascorbic acid-1-$^{14}$C and diketo-L-gulonic acid-1-$^{14}$C to L-ascorbic acid-1-$^{14}$C in guinea pigs.* The isotope dilution technique was particularly necessary for the accurate measurement of the conversion of diketo-L-gulonic to L-ascorbic acid, since an error may be intro-

TABLE 1

*$^{14}C$ in expired $CO_2$ and urine during a 24-hour period following administration of various labeled compounds to guinea pigs*

| Labeled compound | Dose | Route of administration | Fraction of administered $^{14}C$ determined in | | | Avg remaining in animal [1] | Experiments published previously |
|---|---|---|---|---|---|---|---|
| | | | $CO_2$ | Urine | Animal [1] | | |
| | *mg* | | % | % | % | % | |
| L-Ascorbic acid-1-$^{14}C$ | 2.50 | stomach tube | 50 | 3.3 | 47 | | |
| | 2.26 | stomach tube | 35 | 4.4 | 61 | 55 | |
| | 2.41 | stomach tube | 41 | 1.4 | 58 | | |
| | 3.60 | orally by dropper [2] | 40 | 1.6 | 58 | | (4) |
| | 3.60 | orally by dropper [2] | 27 | 3.4 | 70 | 62 | (4) |
| | 2.23 | ip [2] | 26 | 6.3 | 68 | | (4) |
| | 3.32 | ip [2] | 16 | 6.5 | 77 | 71 | (4) |
| | 4.14 | ip [2] | 23 | 9.0 | 68 | | (4) |
| D-Ascorbic acid-1-$^{14}C$ | 1.10 | ip [3] | 28 | 66 | 6 | | (11) |
| | 3.60 | ip [3] | 34 | 52 | 14 | 10 | (11) |
| | 3.52 | stomach tube | 81 | 12 | 7 | | |
| | 3.43 | stomach tube | 78 | 12 | 10 | 8 | |
| Dehydro-D-ascorbic acid-1-$^{14}C$ | 1.06 | ip | 48 | 47 | 5 | | |
| | 1.00 | ip | 53 | 46 | $< 1$ | $< 1$–5 | |
| Dehydro-L-ascorbic acid-1-$^{14}C$ | 3.02 | ip | — | 45 | — | | |
| | 0.84 | ip [4] | 32 | 48 | 20 | 15 | (2) |
| | 1.58 | ip [4] | 38 | 51 | 11 | | (2) |
| | 1.60 | stomach tube | 43 | 6.5 | 51 | | |
| | 1.06 | stomach tube | 51 | 3.7 | 45 | 48 | |
| 2,3-Diketo-L-gulonic acid-1-$^{14}C$ | 4.00 | ip [4] | 29 | 71 | $< 1$ | | (2) |
| | 4.00 | ip [4] | 31 | 68 | $< 1$ | $< 1$ | (2) |

[1] Calculated by difference.
[2] Animals fed a scorbutogenic diet supplemented with 2 mg of vitamin C daily for 2 weeks.
[3] Animals given the Rockland Farm Guinea Pig Diet (Rockland Farms, New City, New York) and water ad libitum.
[4] Feeding schedule similar to present study.

TABLE 2

*Conversion of dehydro-L-ascorbic acid-1-$^{14}$C and 2,3-diketo-L-gulonic acid-1-$^{14}$C to L-ascorbic acid $^{14}$C in intact guinea pigs and rats*

| Pre-treatment | | Compound administered | Route of administration | Dose | Conversion to L-ascorbic acid-1-$^{14}$C at 24 hr (uncorrected) [2] |
|---|---|---|---|---|---|
| | | | | *mg* | % |
| Guinea pig | none | dehydro-L-ascorbic acid-1-$^{14}$C | ip | 1.00 | 20 |
| Guinea pig | none | dehydro-L-ascorbic acid-1-$^{14}$C | ip | 1.00 | 12 |
| Guinea pig | none | dehydro-L-ascorbic acid-1-$^{14}$C | oral | 1.00 | 51 |
| Guinea pig | none | dehydro-L-ascorbic acid-1-$^{14}$C | oral | 1.00 | 68 |
| Rat | 3-methylcholanthrene [1] | dehydro-L-ascorbic acid-1-$^{14}$C | oral | 1.80 | 5.6 [3] |
| Rat | 3-methylcholanthrene [1] | dehydro-L-ascorbic acid-1-$^{14}$C | oral | 1.80 | 5.8 [3] |
| Rat | none | dehydro-L-ascorbic acid-1-$^{14}$C | oral | 1.85 | 4.7 [4] |
| Rat | none | dehydro-L-ascorbic acid-1-$^{14}$C | oral | 1.85 | 4.5 [4] |
| Guinea pig | none | 2,3-diketo-L-gulonic acid-1-$^{14}$C | oral | 4.00 | $< 0.4$ |
| Guinea pig | none | 2,3-diketo-L-gulonic acid-1-$^{14}$C | oral | 4.00 | $< 0.4$ |

[1] 3-Methylcholanthrene (10 mg) was injected intraperitoneally daily for 4 days and the tracer was administered 5 days after the last injection. The body pool of ascorbic acid in 3-methylcholanthrene-treated rats 5 days after the last of 4 such injections was reported to be 225 mg/kg (43). The urinary excretion of vitamin C increased from the control value of less than 1 mg/day to 4 to 25 mg/day.

[2] The accuracy of this data is estimated to be $\pm$ 10%; the largest error is due to the calculation of the body pool. These values can be corrected for metabolism and excretion of L-ascorbic acid. It has been pointed out (14) that "the results of these experiments give minimal values . . . since no correction is made for the amount of labeled L-ascorbic acid metabolized and excreted in urine during the 24-hour period after administration." Obviously the factor is (100%/71%) or 1.4 in the normal guinea pig. Thus the actual reduction of dehydro-L-ascorbic acid-1-$^{14}$C after an oral dose of dehydro-L-ascorbic acid-1-$^{14}$C could be as high as 71 to 85% in guinea pigs. Although the half-life of L-ascorbic acid in the normal rat is about the same as in the guinea pig (3 days), that of 3-methylcholanthrene-treated rats is only 0.85 days (43), leading to a correction of 2.3.

[3] The excretion of $^{14}$C, 24 hours after administration (via stomach tube), was accounted for as 78 and 88% as respiratory $^{14}CO_2$ and 14 and 7%, respectively in urine. Typical for 6 experiments.

[4] The excretion of $^{14}$C, 24 hours after administration (via stomach tube), was accounted for as 84 and 72% of respiratory $^{14}CO_2$ and 11 and 23%, respectively in urine; typical for 7 experiments. One rat given 3 mg dehydro-L-ascorbic acid-1-$^{14}$C intraperitoneally excreted 14% as $^{14}CO_2$ and 47% of $^{14}$C in urine during 24 hours.

TABLE 3

*$^{14}C$ determined in rat liver glycogen following the administration of* L-*ascorbic acid-6-$^{14}C$*

| Pre-treatment [1] | Excretion of L-ascorbic acid the day prior to $^{14}C$ dose | Fraction of dose determined in liver glycogen |
|---|---|---|
| | *mg* | % |
| 3-Methylcholanthrene, 30 mg (in 1.5 ml corn oil), sc | 7.3 | 0.24 |
| | 5.7 | 0.15 |
| | 6.5 | 0.15 |
| | 7.5 | 0.14 |
| Corn oil, 1.5 ml, sc | < 1 | 0.11 |
| | < 1 | 0.10 |
| | < 1 | 0.12 |
| | < 1 | 0.14 |
| | < 1 | 0.16 |

[1] Eight days prior to injection of tracer.

duced upon subtraction of a relatively large number ($^{14}C$ excreted) from another large number (dose). However, it was found that the direct measurements (table 2) were in close agreement with the data in table 1.

Using both methods, it was found that orally administered dehydro-L-ascorbic acid-1-$^{14}C$ was more extensively converted to L-ascorbic acid than upon intraperitoneal injection. No appreciable conversion to diketo-L-gulonic acid-1-$^{14}C$ was observed.

*Studies with 3-methylcholanthrene.* Experiments were carried out in which dehydro-L-ascorbic acid-1-$^{14}C$ was administered to 3-methylcholanthrene pretreated rats. Rats were chosen for these experiments because the effect of pre-treatment on the glucuronic acid pathway can be determined by measuring the increase in urinary excretion of the vitamin. The present data indicate that 3-methylcholanthrene does not markedly increase the extent of reduction of dehydro-L-ascorbic acid in rats. In contrast with the guinea pig, orally administered dehydro-L-ascorbic acid is not extensively reduced in rats (table 2). Furthermore there is no significant change in the conversion of L-ascorbic acid-6-$^{14}C$ to liver glycogen (table 3), despite the increase in biosynthesis of the vitamin in pre-treated animals.

## DISCUSSION

It has been demonstrated that depending upon the route of administration, 30 to 53% of $^{14}C$ of L-ascorbic acid 1-$^{14}C$ administered to guinea pigs was excreted in 24 hours (4), whereas other studies have shown that upon intraperitoneal administration of dehydro-L-ascorbic acid-1-$^{14}C$, 80% of the $^{14}C$ was excreted. In contrast, essentially all of diketo-L-gulonic acid-1-$^{14}C$ was excreted in the same period (2).

On the basis of isotope dilution experiments it was found that in guinea pigs, orally administered dehydro-L-ascorbic acid-1-$^{14}C$ is reduced to L-ascorbic acid-1-$^{14}C$ more extensively than when the tracer is given intraperitoneally. A factor which probably accounts for this difference is the pH range in the stomach as contrasted with that of the peritoneal cavity.

Furthermore, as at least 16% of dehydro-L-ascorbic acid-1-$^{14}C$ is reduced to L-ascorbic acid-1-$^{14}C$, it was estimated that no measurable reduction of diketo-L-gulonic acid -1-$^{14}C$ to dehydro-L-ascorbic acid-1-$^{14}C$ takes place. Because less than 0.4% conversion of diketo-L-gulonic acid-1-$^{14}C$ to L-ascorbic acid occurs, no appreciable (less than 2.4%) conversion of diketo-L-gulonic acid-1-$^{14}C$ to dehydro-L-ascorbic acid-1-$^{14}C$ occurs in guinea pigs.

A scale of "antiscorbutic activity" has been compiled, based upon the percentage of L-ascorbic acid-1-$^{14}C$ present in guinea pigs 24 hours after administration of the following: L-ascorbic acid, intraperitoneal route (71%);[9] L-ascorbic acid, oral route (59%); dehydro-L-ascorbic acid, oral route (48%); dehydro-L-ascorbic acid, intraperitoneal route (15%); 2-keto-gulonic acid, intraperitoneal route (less than 1.3%) (19); and diketo-L-gulonic acid, intraperitoneal route (less than 0.4%).

These results are in agreement with bioassay experiments in which it had been found that oral doses of dehydro-L-ascorbic

[9] Although several studies have been carried out with L-ascorbic acid-1-$^{14}C$ in man (25–28) no studies with dehydro-L-ascorbic acid-1-$^{14}C$ in man have been reported. The results of one study (25) are not in agreement with the others. Criticisms (29) have been raised that the discrepant results may have been due to contamination of the dose with oxidized forms of L-ascorbic acid. Thus, the relatively large excretion of $^{14}CO_2$ reported by these investigators may have been the result of that fraction of dehydro-L-ascorbic acid-1-$^{14}C$ which was not reduced in man, but was oxidized via diketo-L-gulonic acid-1-$^{14}C$ to respiratory $^{14}CO_2$. Recently Schuching and Abt (30) published some data which we interpret as follows: of the ingested L-ascorbic acid, 14% is rapidly oxidized in 24 hours to $^{14}CO_2$. The major portion is incorporated into the L-ascorbic acid body pool, and is slowly metabolized but only to a small extent to $^{14}CO_2$ daily.

acid are nearly as effective in curing scurvy as orally administered L-ascorbic acid (12, 20) and that 2-keto-L-gulonic acid and diketo-L-gulonic acid had no demonstrable antiscorbutic activity (21, 22). Other investigators have shown that subcutaneously administered dehydro-L-ascorbic acid had more activity than orally administered dehydro-L-ascorbic acid in vitamin C-deficient animals (23). The difference between these and the present observations with normal animals may be due to the manner of administration and to impaired mechanisms in the deficient guinea pigs. In humans, the reduction is also influenced by the manner of administration (24).[10] It is therefore of interest that reduction of dehydro-L-ascorbic acid occurs not only in the liver, but also to some extent in the stomach and the intestine of experimental animals (12, 31, 32); reduction by bacteria in the intestines may also play an additional role (33).

At present there is some doubt whether the reduction of dehydro-L-ascorbic acid is entirely non-enzymatic (32, 34). On the other hand the opening of the lactone ring of dehydro-L-ascorbic acid may occur either spontaneously or enzymatically or both (35, 36). Although mono-dehydro-L-ascorbic acid or a complex with L-ascorbic acid (37) may be the most important oxidized intermediate in the metabolism of the vitamin, dehydro-L-ascorbic acid is probably the only one present in useful amounts in the diet. Recently it has been shown that mono-dehydro-L-ascorbic acid is reduced enzymatically (38).

In addition, dehydro-L-ascorbic acid has been demonstrated to be involved in the transport of the vitamin (39). The proposed mechanism is related to the fact that dehydro-L-ascorbic acid is a neutral substance capable of diffusing through lipoid membranes more readily than L-ascorbic acid, which is present mostly in ionized form at physiological pH. However, this passive diffusion process, being non-stereospecific, cannot be of great importance in the renal handling of L-ascorbic acid in the rat. If such a process were the major mechanism involved, D-ascorbic acid would be reabsorbed by the kidney; this is not the case because it was found to be rapidly excreted (11). Dehydro-D-ascorbic acid by being partly reduced, may play a role in contributing to the antiscorbutic activity of D-ascorbic acid. D-Ascorbic acid, despite early reports (22) has been shown to have some antiscorbutic activity (40, 41) and can replace L-ascorbic acid in most but not all in vitro systems (42).

Recently it has been shown that a number of structurally unrelated compounds, including 3-methylcholanthrene (43) stimulate the metabolism and biosynthesis of L-ascorbic acid from D-glucose in the rat. In the present study it was observed that pre-treatment with 3-methylcholanthrene does not significantly alter the conversion of $^{14}C$ from carbon 6 of L-ascorbic acid to glycogen. This information, combined with results of previous studies (2), suggests that intermediate pathways of L-ascorbic acid metabolism are not qualitatively altered by the increased biosynthesis.[11]

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# Influence of Vitamin D on Linear Growth of Normal Full-term Infants [1]

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*ABSTRACT* Because of the possibility that moderate overdosage of vitamin D may interfere with linear growth of human infants, rates of growth and serum concentrations of calcium and inorganic phosphorus were studied in normal full-term male infants fed ad libitum with evaporated milk formulas that supplied either 350 to 550 USP units of vitamin D or 1,380 to 2,170 USP units daily. A group of breastfed male infants receiving 300 USP units of vitamin D daily was also studied. Rates of growth in length and weight and serum concentrations of calcium were similar in the 3 groups. Serum concentrations of inorganic phosphorus were similar in the 2 groups fed evaporated milk; concentrations in these groups were significantly greater than those of the breastfed infants. The study fails to provide evidence that moderate overdosage of vitamin D interferes with rate of growth of normal infants.

Until relatively recently there has been little concern about administering prophylactic doses of vitamin D in the order of 2,000 USP units daily (1). However, reasonable speculation about the relation between doses of vitamin D only several-fold greater than the recommended dietary allowance and occurrence of idiopathic hypercalcemia has led several committees of experts to recommend that daily intakes of vitamin D be restricted at 400 USP units daily from all sources (1, 2).

In addition to the possible relation between moderate overdosage of vitamin D and development of idiopathic hypercalcemia — a relation that presumably pertains to an extremely small percentage of infants — the possibility exists that subtle effects of overdosage may occur in a larger percentage of infants. As long ago as 1938, Jeans and Stearns (3) suggested that intakes of vitamin D in the range of 1,800 to 6,300 USP units daily inhibited linear growth of normal infants.

The present study was designed to explore the influence of relatively slight overdosage of vitamin D on rate of linear growth of normal full-term male infants. Comparison was made between growth rates of infants receiving 350 to 550 USP units of vitamin D daily and those receiving 1,380 to 2,170 USP units daily. This latter dosage range rather than the larger intakes of 1,800 to 6,300 USP units daily mentioned by Jeans and Stearns (3) was chosen because it was believed more representative of amounts likely to be ingested by substantial numbers of normal infants in the United States.

## FEEDINGS

*Evaporated milk feedings.* Two batches of evaporated milk, designated EM-400 and EM-1,600, were employed in the study.[2] These differed only in the extent of fortification with vitamin D, EM-400 providing 400 USP units and EM-1,600 providing 1,600 USP units of vitamin D in each 405-ml can. No other source of vitamin D was permitted.

The evaporated milk was diluted with an equal volume of water before being fed. Carbohydrate was not added. Twenty-five milligrams of ascorbic acid [3] were provided daily beginning at 8 days of age. A preparation of ferrous sulfate [4] supplied 15 mg of elemental iron daily beginning at 30 days of age. Cereal and strained foods commercially prepared for infants and free of vitamin D were permitted according to the following schedule:

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[1] Supported in part by Public Health Service Research grants no. HD-00383, 5 K3 HD-2465, and 5 T1 AM 5246, and in part by a grant from the Evaporated Milk Association.
[2] Provided through the courtesy of the Evaporated Milk Association, Chicago.
[3] Ce-Vi-Sol, Mead Johnson and Company, Evansville, Indiana.
[4] Fer-in-Sol, Mead Johnson and Company, Evansville, Indiana.

28 days — rice cereal
56 days — pears and applesauce
84 days — pears and pineapple, banana
112 days — beets, orange pudding
140 days — meats

Although introduction of the cereal and strained foods was permitted at the ages specified, no attempt was made to encourage the feeding of these foods. Parents of experimental subjects were advised that addition of such foods to the diet was optional and that the milk formula, vitamin preparation and iron constituted a complete diet.

*Breast feeding.* Breastfed infants were permitted one formula feeding [5] daily providing 67 kcal and 50 USP units of vitamin D in 100 ml. Most mothers did, in fact, offer one such formula feeding each day.

Breastfed infants enrolled in the study before March 1965 were permitted cereal and strained foods commercially prepared for infants according to the schedule listed for infants fed evaporated milk. Five infants enrolled after March 1965 did not receive cereal and received strained foods according to the following schedule:

56 days — pears
84 days — applesauce, bananas
112 days — pineapple, beets, carrots
140 days — sweet potatoes, green beans

A vitamin preparation [6] administered from 8 days of age provided the following daily intakes: 1,500 USP units of vitamin A, 200 USP units of vitamin D, 30 mg of ascorbic acid and several vitamins of the B series. A preparation of ferrous sulfate [7] supplied 15 mg of elemental iron daily beginning at 30 days of age.

## SUBJECTS

Normal full-term male infants weighing 2,500 g or more were considered eligible for enrollment in the study. Nearly all were children of students or younger staff members of the State University of Iowa and many were siblings of children who had served as subjects of other studies reported from the Infant Metabolic Unit in recent years. All were enrolled in the program during the first 9 days after birth, nearly all before 4 days of age.

Infants of mothers who expressed a desire to breast feed served as one control group. Other infants were alternately assigned to feeding group EM-400 or feeding group EM-1,600.

## PROCEDURES AND METHODS

Each infant reported to the Metabolic Unit between 6 and 9 days of age for recording of weight and length. Weight was determined to the nearest 5 g on infant scales.[8] Length was measured as described by Falkner (4) utilizing a measuring board with fixed head piece and movable foot piece. Two examiners measured each subject, one holding the head against the headboard while the other stretched the lower extremities, pressed the footboard firmly against the soles of the feet and noted body length. The 2 examiners then exchanged positions and repeated the procedure. When results of the 2 examiners differed by more than 0.4 cm, a third examiner was employed and the 2 values agreeing most closely were utilized.

Measurements of length and weight were repeated at ages 28, 56, 84, 112, 140 and 168 days, in each instance a variation of 4 days from the stipulated age being permitted. Thus, the measurement at age 28 days was made between 24 and 32 days. In computing incremental change in length (or weight), the difference in measurement between one age and the next was divided by the actual number of elapsed days to obtain a value for change in length (or weight) per day.

In the case of infants fed formula EM-400 or EM-1,600, the diet was reviewed with the parent at the time of each visit and an estimate of average daily milk intake during the preceding several days was recorded. It was not possible under the conditions of this study to estimate the volumes of intake of the breastfed infants.

Blood was drawn by external or internal jugular venipuncture once monthly and serum was stored at −20° until determinations of calcium and phosphorus were performed.

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[5] Similac, Ross Laboratories, Columbus, Ohio.
[6] Deca-Vi-Sol, Mead Johnson and Company, Evansville, Indiana.
[7] Fer-in-Sol, Mead Johnson and Company, Evansville, Indiana.
[8] Continental Scales Company, Chicago.

Concentration of calcium was determined fluorometrically as described by Kepner and Hercules (5) except that the reaction was carried out in a polystyrene tube partially immersed in a waterbath at room temperature. Concentration of phosphorus was determined by the method of Fiske and Subbarow (6) as modified by Kaplan and del Carmen (7).

## RESULTS

Two major sources of bias are frequently present in growth studies of human infants. First, the groups of subjects may not be similar at the time of enrollment in the study. Second, failure of some subjects to complete the study may result in significant difference between infants initially enrolled in the study and those providing data suitable for analysis. These possibilities were considered.

*Comparability at time of enrollment.* Since infants were assigned alternately to feeding groups EM-400 and EM-1,600, the possibility that the 2 groups differed significantly at the time of enrollment in the study was considered. As indicated in table 1, mean birth weights, mean lengths and weights at age 8 days and mean parental heights were similar for infants of feeding groups EM-400 and EM-1,600.

Although mean birth weight of breastfed infants was less than mean birth weights of infants in the other 2 feeding groups (table 1), these differences were not statistically significant (8) (mean birth weight of group EM-400 versus that of breastfed infants: $t = 1.43$; $0.2 > P > 0.1$; corresponding values for difference in birth weights of group EM-1,600 and breastfed infants were $t = 1.07$; $P = 0.3$). Such differences probably do not constitute an important source of bias since Meredith (9) has shown from analysis of published data that birth weight and subsequent rate of gain in weight during the first 6 months of life demonstrate a negative correlation of low order. Nevertheless, factors influencing one woman to prefer breast feeding and another to prefer bottle feeding are complex and difficult to evaluate. Selection of infants for enrollment in the group of breastfed infants therefore lacks the random nature possible in assigning bottle-fed infants to one or another bottle feeding.

*Infants failing to complete study.* Figure 1 presents information on early rates of gain in weight of infants who were enrolled in the various feeding groups but failed to complete 112 days of study. Two infants in feeding group EM-400 failed to appear for the visit at 56 days of age and one infant in feeding group EM-1,600 failed to appear for the visit at 84 days of

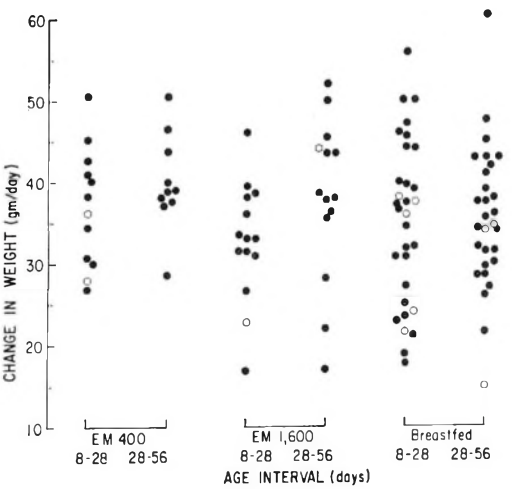


Fig. 1 Early weight gains of all infants enrolled in study. Each solid dot refers to weight gain of one infant who completed at least 112 days of study. Each open circle refers to weight gain of one infant who did not complete 112 days of study (see text).

TABLE 1

*Comparison of infants in 4 feeding groups*

| Feeding group | No. of infants [1] | Mean birth wt | Parental height | | 8-day size | |
|---|---|---|---|---|---|---|
| | | | Mother | Father | Length | Wt |
| | | *g* | *cm* | *cm* | *cm* | *g* |
| EM-400 | 11 | 3,674 | 167 | 177 | 51.6 | 3670 |
| EM-1,600 | 13 | 3,633 | 168 | 179 | 52.2 | 3626 |
| Breastfed | 26 | 3,473 | 165 | 181 | 51.5 | 3430 |

[1] Number completing at least 112 days of study.

age. Reasons for failure to keep the scheduled appointments could not be ascertained.

Of 31 breastfed infants initially enrolled, five failed to complete 112 days of study. After the visit at 28 days of age one family moved out of the state and one mother elected to discontinue breast feeding for reasons not known to the authors. After the visit at 56 days of age one infant was eliminated from the study because the authors encountered exceptional difficulty in obtaining blood by venipuncture, one was eliminated because the mother was unwilling to follow the schedule set for administration of strained foods, and one was lost to follow-up because of failure to appear for subsequent visits. With the exception of this latter infant, who had gained only 15 g/day between 28 and 56 days of age, all infants appeared to be gaining weight normally at the time they were eliminated from the study (fig. 1).

*Estimated daily intakes of milk and vitamin D.* Utilizing information from the monthly reports provided by parents of the infants in feeding group EM-400 or EM-1,600, it was possible to estimate the average daily intake of milk by each infant from time of enrollment until termination of study. Median intake by infants in feeding group EM-400 was 910 ml/day, with a range of 710 to 1,120 ml/day. These intakes provided a median daily dosage of 450 USP units of vitamin D with a range of 350 to 550 USP units. Median intake by infants in feeding group EM-1,600 was 910 ml/day, with a range of 700 to 1,100 ml/day. Median daily dosage of vitamin D was 1,800 USP units with a range of 1,380 to 2,170 USP units.

Breastfed infants received vitamin D from 3 sources: The single formula feeding given to most of the breastfed infants each day afforded an average daily intake of approximately 90 USP units of vitamin D and the daily vitamin supplement provided 200 USP units daily. Because of the exceedingly small amounts of vitamin D provided by human milk (10), total vitamin D intake of the breastfed infants was probably about 300 USP units daily.

*Gains in length and weight.* Data on gains in length and weight are presented in figure 2 and table 2 for the age intervals 8 to 112, 8 to 140 and 8 to 168 days.

Between 112 and 140 days of age no subjects were lost to follow-up in feeding group EM-400 or feeding group EM-1,600. However, four of the 26 breastfed infants who had completed 112 days of study had failed to complete 140 days of study: Two mothers discontinued breast feeding be-

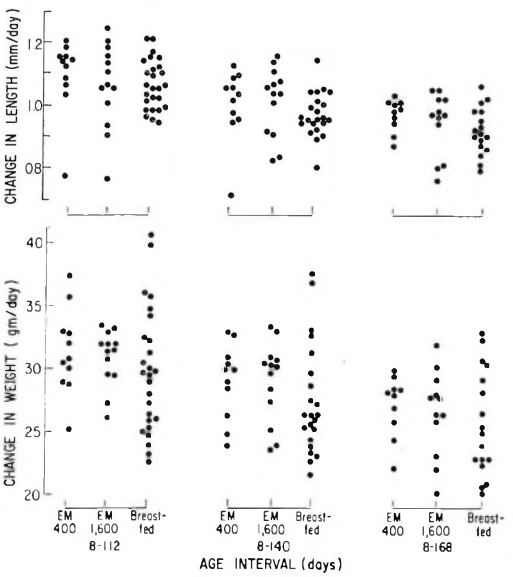


Fig. 2 Change in length and change in weight of infants during the age intervals 8 to 112, 8 to 140, and 8 to 168 days.

TABLE 2
*Gains in length and weight*

| Feeding group | Age interval | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 8–112 days | | | 8–140 days | | | 8–168 days | | |
| | No. of infants | Gain in length | Gain in wt | No. of infants | Gain in length | Gain in wt | No. of infants | Gain in length | Gain in wt |
| | | *mm/day* | *g/day* | | *mm/day* | *g/day* | | *mm/day* | *g/day* |
| EM-400 | 11 | 1.10 | 31.9 | 11 | 1.02 | 29.2 | 10 | 0.99 | 27.8 |
| EM-1,600 | 13 | 1.08 | 31.6 | 13 | 1.03 | 29.5 | 12 | 0.96 | 27.2 |
| Breastfed | 26 | 1.08 | 30.4 | 22 | 0.99 | 28.1 | 17 | 0.94 | 26.8 |

cause they judged their milk supply to be inadequate and two other breastfed infants had not yet reached 140 days of age when the data were assembled for analysis.

Between 140 and 168 days of age, one infant in feeding group EM-400 and one in EM-1,600 were lost to follow-up because the families had moved from town. Of the 22 breastfed infants who had completed 140 days of study, five had failed to complete 168 days of study: Two mothers had discontinued breast feeding because they judged their milk supply to be inadequate; one family had moved from town; for reasons unknown to the authors one infant had failed to appear for the scheduled visit; and one infant had not yet reached 168 days of age.

Mean rates of gain in length and weight were similar for the 3 feeding groups (table 2) although rate of gain in weight (fig. 2) was somewhat more variable among breastfed infants than among those in feeding group EM-400 or EM-1,600.

*Calcium and inorganic phosphorus in serum.* Data on concentrations of calcium and inorganic phosphorus in serum are indicated in table 3. Concentrations of calcium were similar at each age in each feeding group.

Serum concentrations of inorganic phosphorus demonstrated some tendency to decrease with increasing age. The slope of regression of serum inorganic phosphorus differed significantly from zero at the 95% level of confidence (8) in studies with infants in feeding group EM-1,600 ($r = 0.299$) and in studies with the breastfed infants ($r = 0.213$), but not in studies of those in feeding group EM-400 ($r = 0.142$).

At each age the mean concentration of inorganic phosphorus was less for breastfed infants than for infants in feeding group EM-400 or EM-1,600 and the differences were statistically significant at the 95% or 99% level of confidence. The observations are therefore in general agreement with those reported from the Norbotten study (11) for 6- to 9-month-old infants who were breastfed (mean serum concentration of inorganic phosphorus 5.85 mg/100 ml) or received a cow's milk formula (mean serum concentration of inorganic phosphorus 6.22 mg/100 ml).

TABLE 3

*Concentrations of calcium and inorganic phosphorus in sera*

| Age | Feeding group | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | EM-400 | | | EM-1,600 | | | Breastfed | | | |
| | No. of infants | Serum conc | | No. of infants | Serum conc | | No. of infants | Serum conc | |
| | | Ca | P | | Ca | P | | Ca | P |
| *days* | *mg/100 ml* | | | | *mg/100 ml* | | | *mg/100 ml* | |
| 28 | 10 | 9.95 ± 0.65[1] | 7.35 ± 0.60 | 9 | 9.90 ± 0.49 | 7.54 ± 0.70 | 24 | 10.09 ± 0.64 | 6.75 ± 0.65 |
| 56 | 8 | 10.14 ± 0.44 | 7.25 ± 0.61 | 9 | 10.10 ± 0.36 | 7.56 ± 0.26 | 23 | 9.82 ± 0.33 | 6.33 ± 0.68 |
| 84 | 10 | 9.94 ± 0.39 | 7.12 ± 0.42 | 11 | 10.29 ± 0.37 | 7.15 ± 0.48 | 23 | 10.13 ± 0.57 | 6.16 ± 0.50 |
| 112 | 7 | 10.1 ± 0.48 | 7.61 ± 0.62 | 7 | 9.88 ± 0.47 | 7.42 ± 0.37 | 24 | 10.05 ± 0.48 | 6.23 ± 0.65 |
| 140 | 9 | 9.97 ± 0.45 | 7.36 ± 0.70 | 11 | 9.79 ± 0.71 | 6.91 ± 0.63 | 20 | 9.94 ± 0.43 | 6.05 ± 0.57 |
| 168 | 9 | 9.97 ± 0.54 | 6.85 ± 0.60 | 11 | 9.71 ± 0.80 | 7.12 ± 0.36 | 18 | 9.71 ± 0.52 | 6.26 ± 0.20 |

[1] SD.

Concentrations of inorganic phosphorus in serum of infants receiving formula EM-400 or EM-1,600 may be compared with those reported previously (12) for infants receiving similar dietary intakes of protein and phosphorus from a commercially available prepared formula [9] with intakes of vitamin D ranging from 400 to 800 USP units daily. Serum concentrations in the 2 studies were similar during the first 60 days of life (7.6 mg/100 ml in the previous study versus 7.3 and 7.6 mg/100 ml, respectively, for infants fed formula EM-400 and those fed EM-1,600). After 60 days of age, mean concentrations in sera of infants fed formula EM-400 (7.3 mg/100 ml) and EM-1,600 (7.3 mg/100 ml) in the present study were somewhat greater than those reported previously (6.8 mg/100 ml) for infants fed similar dietary intakes of protein and phosphorus. In the present study, mean concentrations of inorganic phosphorus in sera of breastfed infants of various ages were lower and less variable than previously reported (12).

## COMMENT

Results of the present study do not aid in evaluation of the suggestion of Jeans and Stearns (3) that daily intakes of vitamin D in the range of 1,800 to 6,300 USP units may inhibit linear growth of normal infants. However, a mean daily dose of vitamin D of 1,800 USP units, an amount more than 4 times the recommended dietary allowance and one that infants in the United States are much more likely to receive than the doses mentioned by Jeans and Stearns did not interfere with rate of increase in either length or weight. It appears that the majority of normal full-term infants grow at normal rates while receiving moderate overdosage of vitamin D (table 2).

Mean serum concentrations of calcium and inorganic phosphorus were nearly identical among infants receiving evaporated milk providing a mean daily dose of vitamin D of 450 USP units and those receiving evaporated milk providing a mean daily dose of 1,800 USP units. Mean serum concentrations of inorganic phosphorus of breastfed infants were less than those of infants fed evaporated milk.

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[9] Olac, Mead Johnson and Company, Evansville Indiana.

# Hypocholesterolemic Effects Induced in the Rat by Specific Types of Fatty Acid Unsaturation [1,2]

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*ABSTRACT* The comparative effects of specific types of fatty acid unsaturation were tested in adult hypercholesterolemic rats. Test lipid mixtures were adjusted to have iodine values of 40 to 41 and this total unsaturation was supplied by oleate (O), linoleate ($L_2$), linolenate ($L_3$), arachidonate ($A_4$) or a concentrate of eicosapentaenoate and docosahexaenoate ($P_5$-$H_6$). Tallow was the major source of fat calories and $L_2$,$L_3$,$A_4$ and $P_5$-$H_6$ represented, respectively, 7.5, 5.0, 4.1 and 4.0% of the dietary fat. Plasma cholesterol levels continued to rise in the O-fed rats and the palmitate controls, whereas $L_2$ and $L_3$ inhibited further changes in the hypercholesterolemia of the rats. Only the unsaturation from the higher homologues, $A_4$ and $P_5$-$H_6$, exhibited significant hypocholesterolemic effects and lowered myocardial lipid levels. Changes in the distributions of polyunsaturated acids in the heart are apparently more dependent upon the available types of unsaturated acids than the actual pool size of such acids. Exogenous cholesterol, per se, promoted the onset of hypercholesterolemia in adult male Sprague-Dawley rats. Three responses of these rats to treatments with polyunsaturated fatty acids are discussed.

Oils rich in linolenate and its higher homologues [5] have been found to be more effective hypocholesterolemic agents for rats than oils which contain linoleate as the only major source of polyunsaturated fatty acids (PUFA) (1-4). The greater effectiveness of the linolenate family of acids [6] appeared to be a reflection of their greater total unsaturation (1, 4). However, further studies revealed that supplements of the higher homologues of linolenate (supplied as marine oils or their fatty acid ester fractions) were far more effective in alleviating the hypercholesterolemia of rats than could have been predicted on the basis of their total unsaturation or content of total PUFA (2, 5). Such observations suggested the possibility that specific intramolecular arrangements of double bonds in the acids may be playing an important role in determining the relative hypocholesterolemic activities of PUFA observed in natural glycerides and their fatty acid fractions. It seemed desirable to test such a possibility by comparing the hypocholesterolemic activities of different PUFA under conditions in which all of the test lipid mixtures had the same total unsaturation.

The present report describes how the hypercholesterolemia of rats was affected when they received the same total unsaturation from one of the following ethyl ester preparations: oleate, linoleate, linolenate, arachidonate and a concentrate of eicosapentaenoate-docosahexaenoate. Also investigated were the effects of exogenous cholesterol on the circulating lipid levels of rats and the changes in myocardial lipid patterns caused by the different treatments. The 3 different responses promoted by exogenous PUFA in this, and the previous (1, 2, 5), series of studies with hypercholesterolemic rats are discussed.

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[1] Supported by the American Heart Association, Public Health Service Research Grant no. HE-04386, the Minnesota Heart Association and the Hormel Foundation.

[2] Presented, in part, at the annual meeting of the American Heart Association, Cleveland, Ohio, 1962.

[3] These studies were completed during the tenure of an Established Investigatorship from the American Heart Association.

[4] Temporary address: Central Laboratory for Clinical Biochemistry, Karolinska Sjukhuset, Stockholm, Sweden.

[5] Eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids are metabolic products of linolenate 18:3). All 3 homologues are members of the "linolenate family" of acids which have a double bond at the 3-position from the terminal methyl group of the acid:

$$CH_3-CH_2-CH=CH- \ . \ . \ . \ COOH$$

Linoleate (18:2) is the metabolic precursor of arachidonate (20:4) and this "linoleate family" of acids have the double bond at the 6-position from the terminal methyl group:

$$CH_3-CH_2-CH_2-CH_2-CH_2-CH=CH- \ . \ . \ . \ COOH.$$

This latter group is also referred to as the essential fatty acids (EFA) (13).

[6] See footnote 5.

TABLE 1
*Composition of diets*

| | Basal | Basal + cholesterol | Pre-test [1] and test lipid |
|---|---|---|---|
| Casein [2] | 25 | 25 | 25 |
| Cellulose [3] | 4 | 4 | 4 |
| Sucrose | 55 | 54.5 | 54 |
| Salt mix [4] | 4 | 4 | 4 |
| Vitamin mix [5] | 2.1 | 2.1 | 2.1 |
| Beef tallow [6] | 9.9 | 9.9 | 6.9 |
| Cholesterol | 0 | 0.5 | 0.5 |
| Ox bile [7] | 0 | 0 | 0.5 |
| Test lipid mix | 0 | 0 | 3.0 |

[1] Tallow was the only source of fat during pre-test period.
[2] Vitamin-Free, Nutritional Biochemicals Corporation, Cleveland.
[3] Alphacel, Nutritional Biochemicals Corporation.
[4] Mineral mix described by Wesson (26).
[5] Vitamin mix: (mg/100 g diet) ascorbic acid, 5; Ca pantothenate, 4; inositol, 10; niacin, 4; *p*-aminobenzoic acid, 10; riboflavin, 0.5; pyridoxine, 0.2; thiamine·HCl, 0.5; folic acid, 0.2; biotin, 0.05; vitamin $B_{12}$, 0.005; choline·HCl, 100; vitamin A, 0.167; $\alpha$-tocopherol, 5; $\alpha$-tocopheryl acetate, 5; calciferol, 0.002 and menadione, 0.02.
[6] Purified edible tallow, George Hormel Company, Austin, Minnesota.
[7] Powdered preparation, Fisher Scientific Company, Chicago.

TABLE 2
*Test lipid mixtures*

| Ethyl esters | Test lipid added | Palmitate added | Iodine value of mixtures |
|---|---|---|---|
| | *g* | *g* | |
| Palmitate | 0 | 3.00 | 0.0 |
| Oleate | 1.500 | 1.50 | 40.9 |
| Linoleate | 0.750 | 2.25 | 41.2 |
| Linolenate | 0.500 | 2.50 | 41.4 |
| Arachidonate [1] | 0.403 | 2.60 | 41.0 |
| $P_5$-$H_6$ mix [2] | 0.400 | 2.59 | 40.8 |

[1] Contained 5% of longer chain, and trace amounts of shorter chain, fatty acid esters.
[2] Concentrate prepared from the fatty acids of menhaden oil by previously described methods (2). GLC analysis showed that it contained 6.2% monoenoic acids (16:1 and 18:1) and 3.9% of saturated acids ranging in chain length from 14:0 to 24:0. Homologues of linolenate represented 95% of the total PUFA and included: (% of total fatty acids) 18:3, 4; 20:2, 1.8; 20:4, 3.1; 20:5, 33.6; 22:5, 9.1; 22:6, 29.4. The small amounts of linoleate homologues included: (% of total fatty acids) 18:2, 0.7; 20:4, 3.0; 22:5, 1.1.

## EXPERIMENTAL

Adult male rats of the Sprague-Dawley strain weighing 190 to 240 g each, were made hypercholesterolemic by feeding them a diet which contained 10% tallow, 0.5% cholesterol and 0.5% ox bile (table 1). This treatment was continued for 3 months to ensure the establishment of a severe and rather uniform hypercholesterolemic condition in the rats. The rats were then divided into 6 groups having similar plasma cholesterol levels and similar body weights. These groups were fed diets in which 30% of the tallow was replaced by one of the test lipid mixtures shown in table 2. During this test period all groups continued to receive cholesterol and bile acids in their diets, and tallow was the major source of their fat calories.

To test the effects of exogenous cholesterol, per se, one group of rats was maintained with a basal diet containing 10% tallow, and another group received this diet supplemented with 0.5% cholesterol (table 1).

To check for possible subtle differences between the different PUFA, several preliminary experiments were made to establish the minimal levels of linoleate required to elicit a hypocholesterolemic response. Under conditions similar to those described above, linoleate promoted a significant lowering of the plasma cholesterol levels when it supplied the same total unsaturation in the diet as that available from 3 g of oleate. However, when the dietary linoleate was reduced to one-third this level, there was little change in the hypercholesterolemic condition of the rats following a one-month test period. In the study presented here, the test lipid mixtures were adjusted to have the same total unsaturation as that contributed by 1.5 g of oleate and the test period was extended to 2 months. Adjustments of the total unsaturation were accomplished by diluting the different unsaturated esters with palmitate so that all of the test lipid mixtures had iodine values ranging between 40 and 41 (table 2). The palmitate, oleate, linoleate and linolenate were pure ethyl esters.[7] Ethyl arachidonate, a synthetic preparation,[8] served as a source of unsaturation from the higher homologues of linoleate.[9] A concentrate of eicosapentaenoate and docosahexaenoate ($P_5$-$H_6$) was a rich source of the higher homologues of linolenate (see footnotes of table 2).

Cholesterol was analyzed by the method of Abell et al. (6) and phospholipids were determined by a modification of the method of King (7). The gas-liquid

[7] Pure esters purchased from the Hormel Institute, University of Minnesota, Austin, Minnesota.
[8] Kindly supplied by Dr. J. C. Bauernfiend of the Hoffman-LaRoche Company, Nutley, New Jersey.
[9] See footnote 5.

chromatographic (GLC) analyses were performed according to previously described methods of the author (2, 5). Lipids from individual hearts were extracted according to the procedure of Folch et al. (8) and gravimetric analyses were made of pooled samples containing lipids from two to three hearts. The PUFA of individual hearts were analyzed by the alkali isomerization technique described previously (1). This permitted a comparison of PUFA patterns observed in the hearts of rats of this experiment with those obtained in previous studies (1, 2). Both the alkali isomerization and GLC methods have been found to give similar results for the analysis of the major classes of PUFA in dietary lipids and cardiovascular tissues (2). Heart lipids were further fractionated on silicic acid-coated microchromatoplates (2, 9). Quantitative determination of myocardial phospholipids included perchloric acid digestion of the scrapings of specific fractions from microscope slide-microchromatoplates (9). The other analytical methods, precautions taken prior to making heart punctures, and the general experimental design have been described previously (1, 2, 5).

## RESULTS AND DISCUSSION

### *Plasma lipid changes*

When dietary fats contained 33% PUFA as either linoleate or linolenate, both sources of unsaturation were very effective in alleviating the hypercholesterolemia of rats (1). Linolenate, the more unsaturated acid, proved to be more effective than linoleate in the treatment of the hypercholesterolemic condition. In the study reported here, much smaller amounts of these and other acids were tested under conditions in which each of the PUFA contributed the same total unsaturation to the dietary fats (table 2). Oleate, linoleate, linolenate, arachidonate and a concentrate of eicosapentaenoate and docosahexaenoate ($P_5$-$H_6$) represented 15.0, 7.5, 5.0, 4.1 and 4.0% respectively, of the total fat calories derived from unsaturated acids in the test lipid mixtures. The results of such treatments are shown in table 3. The hypercholesterolemia became progressively

TABLE 3

*Effects of cholesterol and unsaturated acid esters on circulating lipids of rats*

| Group | No. of rats | Total plasma cholesterol (TC) | | | Total phospholipids in plasma (TP) | | | Total change [1] in TC + TP | TC/TP |
|---|---|---|---|---|---|---|---|---|---|
| | | Initial | Terminal | Change [2] | Initial | Terminal | Change | | |
| | | *mg/100 ml* | *mg/100 ml* | *%* | *mg/100 ml* | *mg/100 ml* | *%* | *mg/100 ml* | |
| Palmitate | 7 | 497 ± 50 [3] | 872 ± 90 | + 75.2* | 216 ± 17 | 410 ± 37 | + 89.9* | + 569 | 2.1 |
| Oleate | 7 | 468 ± 48 | 718 ± 63 | + 53.5* | 201 ± 18 | 381 ± 43 | + 89.7* | + 430 | 1.9 |
| Linoleate | 7 | 499 ± 43 | 435 ± 50 | − 12.8 | 200 ± 8 | 252 ± 28 | + 26.0 | − 12 | 1.7 |
| Linolenate | 6 | 497 ± 44 | 581 ± 80 | + 16.9 | 201 ± 11 | 300 ± 32 | + 49.3* | + 183 | 1.9 |
| Arachidonate | 7 | 487 ± 49 | 331 ± 32 | − 32.0** | 201 ± 14 | 251 ± 11 | + 25.9** | − 106 | 1.3 |
| $P_5$-$H_6$ | 7 | 482 ± 42 | 314 ± 32 | − 34.8* | 212 ± 16 | 205 ± 14 | − 3.3 | − 175 | 1.5 |
| Basal | 7 | 89 ± 6 | 98 ± 4 | + 10.1 | 137 ± 10 | 168 ± 9 | + 22.6** | + 40 | 0.6 |
| Basal + cholesterol | 6 | 149 ± 26 | 221 ± 33 | + 48.4* | 135 ± 7 | 158 ± 5 | + 14.6** | + 95 | 1.6 |

[1] Total change in TC + TP during terminal 2 months of experiment.
[2] Test of significance by Student's method.
[3] Mean values ± SE of mean.
* Indicates significant change ($P < 0.01$).
** Indicates significant change ($P < 0.05$).

worse in the palmitate controls and the oleate-fed group. The unsaturation derived from the low levels of linoleate and linolenate permitted high plasma cholesterol levels to be maintained in the rats without further significant changes during the test period. However, the same total unsaturation supplied by the double bonds of arachidonate and $P_5$-$H_6$ was sufficient to promote a significant reduction in the plasma cholesterol levels of these 2 groups. Rats fed arachidonate and $P_5$-$H_6$ maintained lower levels of phospholipids (TP), and they had significantly lower levels of TC + TP and lower cholesterol-to-phospholipid ratios (TC/TP) in their plasmas. All groups, except the group fed $P_5$-$H_6$, tended to develop a greater hyperphospholipidemia during the 2-month test period.

Very severe metabolic and dietary imbalances are commonly employed to induce a hypercholesterolemia and atherosclerosis in rats (10). However, during the first 3 months of treatments, exogenous cholesterol caused a 67% increase in plasma cholesterol levels of male Sprague-Dawley rats (table 3). This continued to rise another 48% during the last 2 months of the experiment. There is increasing evidence that exogenous cholesterol may tend to promote similar responses in man (11, 12). The inclusion of both bile acids (cholic acid) and cholesterol in the diet led to the development of a more severe hypercholesterolemia and hyperphospholipidemia in the rats. Following 3 months of treatments with basal, basal + cholesterol and basal + cholesterol + bile acids (the pre-test diet shown in table 1), the rats had average plasma cholesterol levels of 89, 149 and 488 mg/100 ml, respectively.

*Heart lipids and growth responses*

The different treatments had little influence on the phospholipid components in the myocardia of these rats (table 4). All groups had 18 to 21 mg/g of total phospholipids in their hearts, and the relative proportions of sphingomyelin, lecithin, phosphatidyl ethanolamine and phosphatidyl serine (PE + PS) and cardiolipid were similar in the myocardia of all groups. However, the severe hypercholesterolemic condition, which was caused by dietary intakes of bile acid and cholesterol, was accompanied by elevated cholesterol levels in the heart. The palmitate controls and oleate-fed groups had 42 to 50% more cholesterol in their myocardia than that of the basal group. The more mild hypercholesterolemia caused by exogenous cholesterol, per se, (table 3) did not cause any significant change in the sterol or other lipid patterns of the heart. The higher homologues of PUFA promoted 24 to 30% reductions in the heart lipids. Analysis of the pooled samples suggested that this was the result of the lower levels of cho-

TABLE 4

*Heart lipids and growth responses of rats fed cholesterol and unsaturated esters*

| Group | Total [1] lipids | Total phospholipids | Total cholesterol | Terminal [2] body wt |
|---|---|---|---|---|
| | *mg/g tissue* | *mg/g tissue* | *mg/g tissue* | *g* |
| Palmitate | 33 | 21 | 1.7 | 477(44) [3] |
| Oleate | 37 | 19 | 1.8 | 473(38) |
| Linoleate | 33 | 19 | 1.3 | 481(49) |
| Linolenate | 29 | 20 | 1.6 | 468(53) |
| Arachidonate | 23 | 19 | 1.3 | 466(49) |
| $P_5$-$H_6$ | 25 | 18 | 1.2 | 488(61) |
| Basal | 33 | 21 | 1.2 | 464(44) |
| Basal + cholesterol | 30 | 19 | 1.1 | 416(38) |

Myocardial phospholipids: [2] sphingomyelin, 3–8%; lecithin, 33–41%; cephalins (PE+PS), 41–45%; cardiolipid, 13–14.5%.

[1] Values obtained by analyses of 3 pooled samples which contained the lipids from two or three hearts.

[2] Range of values obtained by quantitative TLC analyses of phospholipid components in the hearts of the different groups.

[3] Numbers in parentheses indicate average gain per month during the experimental period.

lesterol and an apparent decrease in the glyceride content in the hearts of rats fed arachidonate and $P_5$-$H_6$.

The different dietary treatments and hypercholesterolemic conditions had little influence on the growth rates of the rats. Rats fed the basal diet had terminal body weights averaging 464 g, and they showed an average gain of 44 g per month during the experimental period (see values in the parentheses of table 4). The other 7 groups exhibited similar growth rates and none of the groups showed any apparent aversion to the different test lipid mixtures.

### *Myocardial PUFA patterns*

In rats and other mammals linoleate is readily converted to arachidonate, and linolenate is the metabolic precursor of the 5- and 6-double-bonded acids such as are present in $P_5$-$H_6$ (13). It seemed possible, therefore, that the limited effects of the unsaturated precursors, linoleate and linolenate, may have been related to some type of deficiency of the different homologues of PUFA in the cardiovascular tissues of the rats. However, the data in table 5 demonstrate that the low intakes of both linoleate and linolenate promoted significant changes in the PUFA patterns of the hearts of the rats, even though these same animals showed no significant changes in their hypercholesterolemic conditions during the test period (table 3). The hearts contained 243 and 455 mg/100 g of linoleate and arachidonate, respectively, in the linoleate-fed group. This was significantly higher than the levels of these same 2 homologues of essential fatty acids (EFA) [10] observed in the myocardia of the palmitate controls. The lower intakes of arachidonate led to even greater changes in the PUFA patterns of the heart. In a similar manner, linolenate promoted the accumulation of appreciable amounts of its higher homologues (the pentaenoates and hexaenoate) into the myocardia of the rats. The group receiving $P_5$-$H_6$ accumulated even greater amounts of the 6-double-bonded acid in their hearts.

In agreement with the observations of Mohrhauer and Holman (14), the more highly unsaturated homologues of PUFA were incorporated into the tissues at the expense of their less unsaturated precursors. This was especially evident in the rats fed arachidonate, where the hearts contained the highest levels of arachidonate (i.e., 636 mg/100 g tissue) but the lowest levels of linoleate (i.e., 27 mg/100 g). In contrast with the latter group, the palmitate controls and oleate-fed rats had 5 to 6 times as much linoleate in their myocardia. The low ratios of trienoates to arachidonate (i.e., 0.2 or less) in the myocardia suggested that all groups had received adequate supplies of EFA during the course of the experiments (15, 16).

### *PUFA pool sizes and myocardial lipid patterns*

Previous studies (2) revealed that rats fed widely different concentrations of $P_5$-$H_6$ accumulated very similar levels of 5- and 6-double-bonded acids in their hearts and circulating phospholipids. Although the 2 different tissues had their own characteristic patterns of PUFA, the plasma and heart phospholipids had similar levels of pentaenoates and hexaenoate whether

[10] See footnote 5.

TABLE 5

*Effects of unsaturated esters on patterns of polyenoic acids in the heart* [1]

| Group | 18:2 | 20:3–18:3 | 20:4 | 20:5–22:5 | 22:6 | Total PUFA |
|---|---|---|---|---|---|---|
| | mg/100 g *tissue* | mg/100 g *tissue* | mg/100 g *tissue* | mg/100 g *tissue* | mg/100 g *tissue* | *mg/g tissue* |
| Palmitate | 167 ± 7 [2] | 79 | 369 ± 27 | 50 ± 5 | 113 ± 7 | 7.8 |
| Oleate | 119 ± 7 | 62 | 375 | 49 | 119 | 7.2 |
| Linoleate | 243 ± 31 | 0 | 455 ± 20 | 59 | 102 | 8.6 |
| Linolenate | 129 ± 8 | 28 | 261 ± 9 | 122 ± 6 | 218 ± 6 | 7.6 |
| Arachidonate | 27 ± 8 | 0 | 636 ± 34 | 74 ± 7 | 71 ± 7 | 8.1 |
| $P_5$-$H_6$ | 99 ± 19 | 0 | 202 ± 25 | 94 ± 8 | 302 ± 30 | 7.0 |

[1] Results obtained by the alkali isomerization method (see Experimental section).
[2] Mean values ± SE of the means are recorded where significant differences between the palmitate controls and other groups were indicated.

the rats received trace amounts or 33% of their fat calories in the form of $P_5$-$H_6$. This apparent homeostatic regulation of tissue PUFA is further illustrated in table 6. The data show a comparison of results obtained in the present study with those of previous experiments (1, 2). In every case, the rats were subjected to similar experimental conditions and the tissues were analyzed by similar methods. The data show that rats fed 7.5 to 33.3% of their fat calories as linoleate, in 3 different experiments, accumulated similar levels of linoleate, arachidonate and total PUFA in their hearts. Dietary intakes of widely different levels of linolenate or its higher homologues ($P_5$-$H_6$) led to the establishment of a different pattern of PUFA in the heart, but the levels of arachidonate, pentaenoates and hexaenoate were essentially constant and independent of the available supplies of PUFA in the rats. Such constancy of tissue PUFA is probably closely related to the specific fatty acid patterns which are established in cardiovascular tissues during the biosynthesis of phospholipids (17). Other recent studies by this investigator [11] have revealed that the fatty acid patterns noted in cardiolipids of rats and pigs are also more dependent upon the available types of PUFA than the total pool of PUFA.

## GENERAL DISCUSSION

Studies with human subjects (18) and experimental animals (1, 4) have often suggested that the hypocholesterolemic effects of oils are merely reflections of the total unsaturation made available by their PUFA components. However, this and the previous reports of these investigators (1, 2, 5) have demonstrated a close relationship between the availability of specific types of PUFA in an oil and its potential effectiveness as a hypocholesterolemic agent. Results of the present study have revealed that the total unsaturation from the higher homologues of both the linoleate and linolenate families of acids [12] have more hypocholesterolemic activities than those of their less unsaturated precursors. The unsaturation from the 4 double bonds of arachidonate was far more effective in alleviating the hypercholesterolemia of rats than was the same total unsaturation supplied by double the molecular concentration of linoleate (tables 2 and 3). A similar relationship was also apparent between the effects of linolenate and its higher homologues present in $P_5$-$H_6$. Such observations strongly suggest that

[11] Peifer, J. J. 1965 Cardiolipids of the heart and liver of pigs and rats. Presented at 9th Internat. Conference on the Biochemistry of Lipids, Sept. 5, at Noordwijk, Netherlands.

[12] See footnote 5.

TABLE 6

*Effects of caloric intakes of PUFA on myocardial lipid patterns* [1]

| Exogenous PUFA | 18:2 | 18:3 | 20:4 | 20:5–22:5 | 22:6 | Total PUFA | References |
|---|---|---|---|---|---|---|---|
| *% of fat cal* | *mg/100 g tissue* | *mg/100 g tissue* | *mg/100 g tissue* | *mg/100 g tissue* | *mg/100 g tissue* | *mg/g tissue* | |
| Linoleate | | | | | | | |
| 33.3 [2] | 332 | 0 | 503 | 75 | 65 | 9.8 | (1) |
| 18.9 [3] | 378 | 0 | 478 | 77 | 118 | 10.5 | (2) |
| 7.5 [2] | 243 | 0 | 455 | 59 | 102 | 8.6 | (see table 5) |
| Linolenate | | | | | | | |
| 33.3 [2] | 62 | 57 | 215 | 175 | 207 | 7.2 | (1) |
| 5.0 [2] | 129 | 28 | 261 | 122 | 218 | 7.6 | (see table 5) |
| $P_5$-$H_6$ | | | | | | | |
| 27.0 [2] | 83 | 0 | 226 | 168 | 387 | 8.6 | (2) |
| 25.0 [3] | 91 | 0 | 346 | 176 | 507 | 9.7 | (1) |
| 4.0 [2] | 99 | 0 | 202 | 94 | 302 | 7.0 | (see table 5) |
| 2.0 [2] | 132 | 0 | 258 | 138 | 355 | 8.6 | (2) |

[1] Heart lipids from rats fed the tallow-cholesterol-bile acid diet (see table 1) in 3 different experiments over the past 5 years.

[2] PUFA fed as ethyl esters.

[3] PUFA supplied as corn oil or marine oil.

the endogenously formed higher homologues of PUFA make significant contributions to the hypocholesterolemic effects initiated by dietary intakes of oils rich in linoleate or linolenate, or both. The presence of specific types of PUFA also appears to be more important than the lack of saturated and monoenoic acids in these experiments. Hypocholesterolemic effects have been evident in rats fed fats which contained 90 to 95% saturated + monoenoic acids and little more than trace amounts of the higher homologues of PUFA (2, 5) tables 2 and 3).

Under the conditions of this series of experiments, 3 different responses have been demonstrated in hypercholesterolemic rats fed polyunsaturated oils: 1) a change in the PUFA patterns of the cardiovascular tissues, 2) a hypocholesterolemic response, and 3) a mobilization of cholesterol from the livers of the rats. The magnitude of the responses depends upon both the available supply of PUFA and the availability of specific types of polyenoic acids. The patterns of polyunsaturated acids established in some cardiovascular tissues are much more dependent upon the available types of PUFA than upon the total available supply of PUFA. However, both the amount of total PUFA and the availability of specific homologues of PUFA influences the hypocholesterolemic responses initiated in these animals. The mobilization of cholesterol from the liver also appears to depend upon the amounts and types of PUFA made available to the rats. The latter response has been observed only in rats that had been treated for 6 to 7 weeks with oils which contained appreciable amounts of eicosapentaenoate and docosahexanoate (2, 5). Although arachidonate had not been tested in these studies (2, 5), results of the experiment reported in this paper suggest that this higher homologue of linoleate [13] would also be effective in the mobilization of cholesterol from the liver. However, equal caloric intakes of linoleate have had little influence on the liver lipids of these rats (2), even though such treatment promoted a significant hypocholesterolemic response in the animals. The maximal positive response from treatments with polyunsaturated oils may depend upon a balance between different types of PUFA more than the high intakes of any single polyunsaturated acid (i.e., linoleate) or only one family of PUFA.[14]

Although the unsaturated acids have been reported to enhance the absorption of cholesterol (19, 20), other observations have suggested that linolenate-rich oils may promote a transient hypercholesterolemia followed by an increased elimination of the sterol from the cardiovascular tissues (21). Some experimental studies have also suggested that the higher homologues of linolenate may actually interfere with the absorption of cholesterol (22). The difficulties of establishing ideal experimental conditions for cholesterol balance studies have been emphasized in the recent reports of Spritz et al. (23). Much of the related research on this subject (24) has not included the carefully controlled conditions needed to relate plasma cholesterol changes with the excretion of sterols and bile acids. Even less attention has been given to the possible role of specific types of PUFA on the sterol and steroid excretion by normal and hypercholesterolemic animals. The effects of PUFA described in this and the previous reports (1, 2, 5) have occurred in animals that continued to receive comparatively high intakes of cholesterol and bile acids. This must mean that even low intakes of some types of PUFA, such as arachidonate and $P_5$-$H_6$, are capable of counteracting the influence of the cholesterol-bile acid double feedback phenomenon described by Beher et al. (25).

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[13] See footnote 5.
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