Essential Fatty Acid Deficiency and the Testis: Lipid Composition and the Effect of Preweaning Diet [1]

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ABSTRACT These experiments were designed to characterize the lipid changes in the rat testis as essential fatty acid (EFA) deficiency progresses and to relate these to the gross and histological state of the tissue. Also, information was sought as to why reports of testis damage in EFA deficiency have been conflicting. It was found that predepletion by feeding an EFA-deficient diet 11 days before weaning resulted in testis damage in 6 to 9 weeks compared with more than 16 weeks in rats fed the diet after weaning. Three percent of saturated fat did not accelerate EFA deficiency compared with a fat-free diet. Analyses of testes for total lipid, total phospholipid, phospholipid classes, total fatty acids and fatty acids in the choline and ethanolamine phosphatides after 6 and 9 weeks of EFA deficiency did not reveal any changes which could be correlated with the onset of histological damage. The testis appears to be particularly sensitive to EFA deficiency during the developmental period, but if maturation occurs, then a prolonged period of deficiency is required before damage appears. The results suggest that secondary effects of EFA deficiency are responsible for eventual testis degeneration.

Reports of the effects of essential fatty acid (EFA) deficiency on the rat testis are conflicting. Although the original observation of Burr and Burr (1) that EFA-deficient rats had small testes and were sterile has been confirmed numerous times (see Aaes-Jørgensen (2) for a review), there are several reported exceptions. Mackenzie et al. (3) found normal testes in rats after 44 weeks on a low fat diet, and Greenberg and Ershoff (4) obtained similar results after 21 weeks of EFA deficiency. Panos and Finerty (5) reported normal testis weights but microscopic evidence of tubular degeneration after 20 weeks of deficiency. Aaes-Jørgensen et al. (6) found testicular degeneration in rats fed high levels of saturated fat but testes from rats given a fat-free diet for 18 weeks were normal. In subsequent work (2), these authors noted that rats depleted of EFA for 26 weeks with a diet containing 7% or 28% of trilaurin had testes with widely varying histological pictures; some were normal whereas others showed severe degeneration.

Our first experiments on the EFA-testis relationship were in accord with the above exceptions, namely, rats deficient for 26 or more weeks had normal testes. The present report describes experiments designed to characterize the lipid changes in the testis as EFA deficiency progresses and to relate these to the gross and histologic state of the tissue. In addition, the effects of preweaning depletion of EFA in accelerating testicular degeneration are described.

EXPERIMENTAL

Weanling male rats of the Holtzman strain,[2] weighing 45 to 55 g, or rats from the National Institutes of Health colony (Sprague-Dawley strain) were used. They were caged individually in stainless steel suspended cages and given food and water ad libitum. Room temperature was 24 ± 1° and relative humidity varied from 33 to 50%. The percentage composition of the basal fat-free diet, R-11F, was: vitamin free casein,[3] 22; salt mixture (7), 6; vita-

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[1] A preliminary report of this study was presented at the meeting of the American Oil Chemists Society, April 24–27, 1966, Los Angeles, California.

[2] Holtzman Company Animal Breeders, Madison, Wisc.

[3] Nutritional Biochemicals Corporation, Cleveland, Ohio.

min mixture,[4] 2; and sucrose, 70. When fat was added to the diets it replaced an equal weight of sucrose. The fats used and their content of linoleic acid (as percentage of total fatty acids) were: corn oil,[5] 55; hydrogenated coconut oil,[6] less than 0.5; and trilaurin,[7] less than 0.5.

Testes were frozen at $-20°$ until analyzed. Chloroform–methanol extracts were prepared by the method of Folch et al. (8) and aliquots of the extracts were analyzed for total lipid, total cholesterol and phospholipid as described previously (9). Phospholipid classes were separated by the thin-layer chromatographic procedure of Parker and Peterson (10) but with a solvent mixture of chloroform–methanol–acetic acid–water in ratios of 100:60:14:7, respectively. The various zones were located with iodine vapor; the silica gel was scraped off into a tube and phosphorus determined as described by Parker and Peterson (10). Standards for lecithin, cephalin and lysolecithin were obtained commercially.[8] A pure sphingomyelin sample was supplied by Dr. J. N. Kanfer of the National Institute of Neurological Diseases and Blindness. Fatty acids were determined for the total lipid extracts and for two phosphatide fractions. Because the amounts of lysolecithin, sphingomyelin and the combined serine plus inositol phosphatides obtained from individual testes were very small, fatty acid data were obtained only for the choline and ethanolamine phosphatides.

Fatty acids were methylated using 1 ml methanol, 0.5 ml benzene and 0.1 ml BF_3 for 30 minutes at 65°. The methyl esters were analyzed on a gas chromatograph[9] with an argon ionization detector (11). The column was 4 mm by 180 mm packed with 15% ethylene glycol succinate on Chromosorb W, 80 to 100 mesh. Temperatures were: column, 180°; detector, 225°; and flash heater, 240°. Peak areas were calculated by triangulation, and identification was made with National Institutes of Health standards or, for long-chain polyunsaturated acids, by comparison of carbon numbers provided by Dr. R. L. Holman.[10] Chromatographic runs were stopped after $C_{22:5\omega6}$ appeared. For the phospholipid fatty acids, the various phosphatide zones on the thin-layer plates were visualized by spraying with 2,6-dichlorofluorescein, the silica gel transferred to a tube and the methylation performed directly as described above. For histological examination testes and epididymes were fixed in Bouin's solution and sections stained by the periodic acid-Schiff procedure.

RESULTS

In all experiments, rats fed EFA-deficient diets exhibited the classic symptoms of scaly feet and tails by weeks 10 to 12. Occasional checks of water consumption showed that the deficient rats had higher intakes than the control animals (1). The most definitive evidence, however, of EFA deficiency is the change in fatty acid composition and these data are given for each experiment.

Experiment 1. In this experiment 3% trilaurin was added to the basal EFA-deficient diet and animals were killed after 7 and 22 weeks. Testes of the EFA-deficient rats were normal in appearance and size (table 1) even after 22 weeks. No histology was done on these testes. Fatty acid analyses revealed the typical pattern of rats deprived of linoleic acid, that is, a marked decrease in fatty acids ($\omega6$) derived from linoleate and an increase in acids ($\omega9$) synthesized from oleate.

Experiment 2. The effects of two different saturated fats in the EFA-deficient diet were compared in this experiment which was continued for 34 weeks. One group received the basal, fat-free diet, one group was fed this diet with 3% trilaurin and another group received 3% hydrogenated coconut oil. A control group had 3% corn oil in the diet. Two to four rats were killed after 9, 16, 26 and 34 weeks. In addition to the testes, livers were also taken for fatty acid analyses to verify the degree of EFA deficiency. Triene-to-tetra-

[4] The vitamin mixture provided in milligrams per kilogram diet: thiamine, 15; riboflavin, 15; pyridoxine, 15; calcium pantothenate, 45; niacin, 50; choline chloride, 1000; folic acid, 2; biotin, 1; vitamin B_{12}, 0.03; viamin A acetate (stabilized), 6; *dl*-α-tocopheryl acetate, 200; vitamin D_3, 0.075; 2-methyl,1,4-naphthoquinone, 1. Selenium (0.1 mg/kg) was also added as sodium selenite.

[5] Mazola, Best Foods, Division of Corn Products Company, New York, N. Y.

[6] Hydrol, Durkee Famous Foods, New York, N. Y.

[7] Drew Chemical Corporation, Boonton, N. J.

[8] Mann Research Labs., Inc., New York, N. Y.

[9] Barber-Colman Model 15, Barber Colman Company, Rockford, Ill.

[10] Personal communication.

TABLE 1

Weight and lipid composition of testes from rats fed an essential fatty acid deficient-diet with trilaurin [1]

	Basal		Basal + 0.5% methyl linoleate [2]	
No. of weeks	7	22	7	22
No. of rats	2	3	3	3
Body wt, g	263 ± 22	378 ± 24	273 ± 13	425 ± 16
Testes wt, g	3.08 ± 0.00	3.36 ± 0.09	3.02 ± 0.18	3.43 ± 0.12
Total lipid, mg/g	22.6 ± 1.7	22.8 ± 0.8	22.6 ± 0.8	21.1 ± 0.8
Phospholipid, mg/g	12.8 ± 0.1	12.0 ± 0.2	12.9 ± 0.4	11.0 ± 0.1
Cholesterol, mg/g [3]	2.1 ± 0.1	—	2.1 ± 0.2	—
Fatty acids [4]				
$C_{16:0}$	35.3 ± 0.6	30.9 ± 1.6	34.2 ± 0.9	31.2 ± 0.6
$C_{16:1}$	4.3 ± 1.7	6.3 ± 1.2	2.9 ± 0.5	3.8 ± 0.4
$C_{18:0}$	5.3 ± 0.4	4.8 ± 0.1	5.1 ± 0.4	5.0 ± 0.3
$C_{18:1}$	24.3 ± 2.3	28.0 ± 1.0	20.0 ± 1.7	18.7 ± 0.9
$C_{18:2}$	0.9 ± 0.0	0.5 ± 0.0	2.4 ± 0.1	2.3 ± 0.1
$C_{20:3\omega9}$	6.1 ± 0.5	8.4 ± 0.2	—	—
$C_{20:4\omega6}$	7.4 ± 1.5	5.6 ± 0.6	12.8 ± 0.9	14.3 ± 0.4
$C_{22:3\omega9}$	1.7 ± 0.1	1.6 ± 0.1	—	—
$C_{22:4\omega9}$	2.2 ± 0.2	3.5 ± 0.1	—	—
$C_{22:4\omega6}$	—	0.9 ± 0.2	1.4 ± 0.1	1.5 ± 0.1
$C_{22:5\omega6}$	8.1 ± 2.1	6.3 ± 0.6	16.8 ± 1.3	20.3 ± 1.1

[1] Weanling rats of the Holtzman strain fed diet R-11F plus 3% trilaurin. Values are means ± SE.
[2] From Hormel Institute, Austin, Minn. Contained 0.02% butylated hydroxytoluene as antioxidant.
[3] No determinations were made on the 22-week samples.
[4] Expressed as percentage of total fatty acids. Acids uniformly less than 1% are not reported. Blank values indicate less than 0.2%. Acids designated ω6 are derived from linoleic acid and ω9 from oleic acid.

ene ratios of 2–4 in the livers (table 2) are similar to those reported for heart and blood of severely EFA-deficient rats (12).

To conserve space, detailed fatty acid data for the testes at 16, 26 and 34 weeks only are given (table 3). A general fatty acid pattern was established in the three EFA-deficient groups by 16 weeks; namely, elevated $C_{16:1} + C_{18:1}$, $C_{20:3\omega9}$, $C_{22:3\omega9}$ and $C_{22:4\omega9}$, and decreased $C_{18:2\omega6}$, $C_{20:4\omega6}$ and $C_{22:5\omega6}$. This pattern did not change appreciably during the next 18 weeks; in general, the changes were only slightly accentuated. Feeding of trilaurin did not change the fatty acid picture from that of the fat-free group, but hydrogenated coconut oil gave slightly higher levels of some ω6 acids and lower amounts of ω9 acids.

Testes taken after 9, 16 and 26 weeks were normal in weight in terms of body weight and were histologically normal. By 34 weeks, however, the testes of all EFA-deficient rats were subnormal in weight and histologically showed mild degrees of injury. There was thinning of the germinal epithelium and sloughing of germ cells in many seminiferous tubules, and there were reduced numbers of sperm intermingled

TABLE 2

Ratio of trienoic-tetraenoic acids in total fatty acids from liver of essential fatty acid-deficient rats [1]

Diet supplement	Weeks [2]		
	16	26	34
None	2.6	2.8	2.7
3% trilaurin [3]	2.8	3.1	4.1
3% hydrogenated coconut oil [4]	2.4	— [5]	2.2
3% corn oil	0.02	— [6]	— [6]

[1] Holtzman rats were fed basal diet R-11F with supplements indicated from weaning. There were nine rats in each group except those fed hydrogenated coconut oil; this group had six rats.
[2] Livers from two to four rats analyzed at each period. Values are means ± SE of the ratios $C_{20:3\omega9}/C_{20:4\omega6}$.
[3] Fatty acid composition: $C_{12:0}$, 99%; $C_{14:0}$, 1%.
[4] Fatty acid composition: $C_{10:0}$, 5%; $C_{12:0}$, 47%; $C_{14:0}$, 22%; $C_{16:0}$, 12%; $C_{18:0}$, 11%; and $C_{18:1}$, 3%.
[5] No analyses made.
[6] $C_{20:3\omega9}$ was not measurable.

TABLE 3

Effect of saturated fat on testis fatty acid composition in essential fatty acid deficiency [1]

Dietary fat	No. of weeks	No. of rats	Body wt [2]	Testes wt	$C_{16:0} + 18:0$	$C_{16:1} + 18:1$	$C_{18:2}$	$C_{20:3\omega9}$	$C_{20:4\omega6}$	$C_{22:3\omega9}$	$C_{22:4\omega9}$	$C_{22:4\omega6}$	$C_{22:5\omega6}$
			g	*g*									
None	16	3	372 ± 11	2.94 ± 0.06	37.0 ± 0.9	27.9 ± 1.6	0.5 ± 0.1	8.3 ± 0.2	7.0 ± 0.5	2.0 ± 0.1	3.2 ± 0.1	0.7 ± 0.1	8.0 ± 0.4
	26	2	410 ± 9	3.14 ± 0.28	39.4 ± 0.8	26.8 ± 1.6	0.4 ± 0.0	9.5 ± 0.1	7.0 ± 0.6	2.0 ± 0.1	3.9 ± 0.0	—	7.0 ± 1.1
	34	3	421 ± 15	2.69 ± 0.25	39.4 ± 0.3	30.7 ± 0.3	0.5 ± 0.0	9.7 ± 0.5	6.2 ± 0.5	2.0 ± 0.1	3.7 ± 0.2	0.8 ± 0.1	5.5 ± 0.2
3% trilaurin	16	3	404 ± 10	3.12 ± 0.14	38.2 ± 0.7	24.1 ± 0.7	0.6 ± 0.0	8.8 ± 0.1	7.5 ± 0.1	2.1 ± 0.1	3.3 ± 0.1	0.6 ± 0.1	8.8 ± 0.2
	26	2	413 ± 15	3.67 ± 0.17	37.7 ± 1.3	30.1 ± 1.7	0.4 ± 0.0	9.5 ± 0.7	6.0 ± 0.1	1.9 ± 0.2	3.5 ± 0.3	—	6.4 ± 0.8
	34	4	420 ± 18	2.62 ± 0.12	40.5 ± 0.6	27.0 ± 1.0	0.5 ± 0.0	10.0 ± 0.2	5.5 ± 1.0	2.1 ± 0.2	3.8 ± 0.1	0.6 ± 0.0	6.8 ± 0.4
3% hydrogenated coconut oil	16	2	378 ± 4	3.22 ± 0.10	41.5 ± 0.3	26.6 ± 0.5	0.6 ± 0.0	6.9 ± 0.2	6.9 ± 0.2	1.7 ± 0.0	2.5 ± 0.2	0.6 ± 0.1	9.3 ± 1.1
	26 [3]	–	399 ± 4	—	—	—	—	—	—	—	—	—	—
	34	2	439 ± 4	3.14 ± 0.11	39.6 ± 0.4	28.8 ± 0.4	0.6 ± 0.2	7.5 ± 1.3	6.9 ± 0.6	1.7 ± 0.2	2.9 ± 0.3	0.5 ± 0.0	8.2 ± 0.4
3% corn oil	16	3	431 ± 17	3.03 ± 0.15	40.7 ± 0.7	17.2 ± 0.3	4.3 ± 0.2	0.0	13.7 ± 0.6	0.0	0.0	1.7 ± 0.1	18.8 ± 0.8
	26	2	469 ± 20	3.53 ± 0.01	35.2 ± 0.6	23.3 ± 1.9	5.7 ± 1.0	0.0	13.3 ± 0.6	0.0	1.5 ± 0.0	0.0	17.3 ± 1.7
	34	4	526 ± 20	3.65 ± 0.10	38.3 ± 0.7	19.5 ± 0.9	4.6 ± 0.4	0.0	14.5 ± 0.5	0.0	0.0	1.8 ± 0.0	17.4 ± 0.9

[1] See footnote 1, table 2 for experimental details. See footnote 4, table 1 for explanation of values.
[2] Average weight of animals remaining at each time period.
[3] No animals killed at this period.

with sloughed germ cells in the ducts of the caput epididymis. These changes were most evident in rats of the fat-deficient group.

Experiment 3. Because only a mild degree of testicular damage occurred after 26 weeks of EFA deficiency in experiments 1 and 2, an attempt was made to accelerate the deficiency state by predepleting young rats prior to weaning. Lactating stock females and their suckling young, obtained from the NIH Animal Production Section, were fed the basal fat-deficient diet (R-11F) from days 14 to 21 of lactation. The 21-day weanlings were fed the diet an additional week and then were grouped and fed the same diet, with or without additions of 3% hydrogenated coconut oil or corn oil. After 26 weeks, testes of all EFA-deficient rats were in advanced stages of degeneration and caput epididymes were devoid of sperm. Lesser degrees of injury were observed after 20 weeks (data not shown).

Experiment 4. The preliminary experiment recorded above was repeated with transfer of mothers and litters to the EFA-deficient diet on day 10 of lactation rather than day 14 as before. In addition to the predepleted rats, a group of rats whose mothers were maintained continuously on the stock diet were started on the EFA-deficient diet when weaned (designated "conventional EFA-deficient" rats).

Growth of both EFA-deficient groups was similar, and average body weights were about 89% of that of the corn oil controls after 9 weeks (table 4). Both deficient groups had scaly feet by week 5; scaliness of the tail was apparent after 5 weeks only in predepleted rats but was marked in both groups by 9 weeks.

After only 6 weeks, testes of predepleted rats were definitely subnormal in weight

TABLE 4

Weight and lipid composition of testes from conventional or predepleted essential fatty acid-deficient rats

	Predepleted EFA deficient [1]		Conventional EFA deficient [2]		3% corn oil controls [3]	
No. of weeks	6	9	6	9	6	9
No. of rats	15	10	19	14	11	9
Body wt, g	217 ± 5	288 ± 8	205 ± 6	267 ± 12	237 ± 9	312 ± 12
No. of rats analyzed [4]	5	3	5	3	2	2
Testes wt, g	1.96 ± 0.19	2.54 ± 0.48	2.88 ± 0.05	3.00 ± 0.07	3.22 ± 0.16	3.48 ± 0.07
Total lipid, mg/g tissue	18.6 ± 0.5	19.6 ± 0.6	18.0 ± 0.6	17.7 ± 0.3	17.6 ± 0.8	17.4 ± 0.7
Phospholipid, mg/g tissue	13.9 ± 0.3	13.9 ± 0.4	14.4 ± 0.2	13.6 ± 0.0	14.8 ± 0.5	13.3 ± 0.6
Fatty acids [5]						
$C_{16:0}$	34.3 ± 0.9	36.3 ± 1.7	37.5 ± 1.7	33.9 ± 2.9	32.8 ± 0.2	32.7 ± 0.3
$C_{16:1}$	1.4 ± 0.2	1.7 ± 0.3	1.4 ± 0.2	1.5 ± 0.2	0.5 ± 0.0	0.5 ± 0.1
$C_{18:0}$	7.3 ± 0.3	7.1 ± 0.4	6.4 ± 0.5	6.8 ± 0.5	7.0 ± 0.4	7.1 ± 0.1
$C_{18:1}$	25.7 ± 0.5	23.2 ± 1.5	25.0 ± 1.1	23.7 ± 1.5	14.1 ± 0.6	13.3 ± 0.2
$C_{18:2}$	0.7 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	3.5 ± 0.1	3.3 ± 0.1
$C_{20:3\omega9}$	9.4 ± 0.5	10.0 ± 0.5	7.4 ± 0.1	8.3 ± 0.5	—	—
$C_{20:4\omega6}$	7.5 ± 0.2	7.1 ± 0.2	8.3 ± 0.5	8.7 ± 0.7	18.1 ± 0.1	18.5 ± 0.2
$C_{22:3\omega9}$	2.3 ± 0.1	2.1 ± 0.1	1.8 ± 0.2	1.7 ± 0.1	—	—
$C_{22:4\omega9}$	3.2 ± 0.2	3.1 ± 0.6	2.2 ± 0.2	2.9 ± 0.2	—	—
$C_{22:4\omega6}$	—	—	—	—	1.8 ± 0.0	1.7 ± 0.0
$C_{22:5\omega6}$	6.2 ± 0.5	7.1 ± 0.4	6.9 ± 0.9	9.7 ± 0.9	19.6 ± 0.8	19.4 ± 0.6

[1] Fed EFA-deficient diet R-11F when 10 days old; weaned at 21 days.
[2] Fed EFA-deficient diet R-11F when weaned at 21 days of age.
[3] Fed diet R-11F plus 3% corn oil when weaned at 21 days of age.
[4] No cholesterol analyses were made.
[5] See footnote 4, table 1.

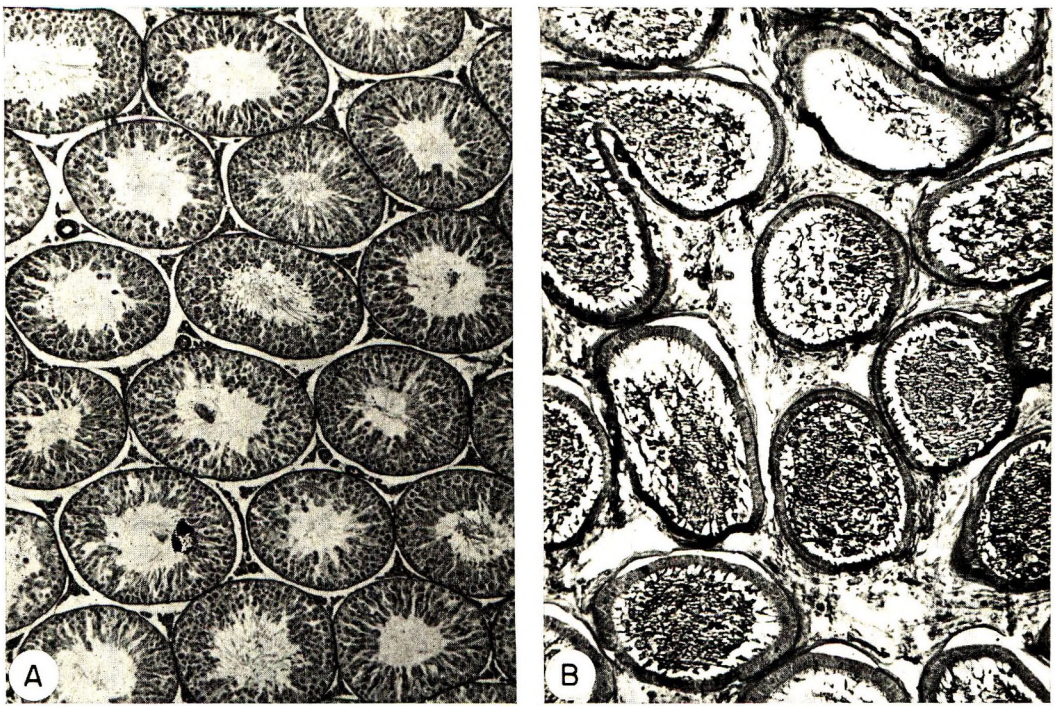

Fig. 1 (*A*) Testis from rat predepleted of EFA. Tissue removed 6 weeks after weaning. Tubules are small with broken epithelium. PAS × 74. (*B*) Caput epididymis from testis in figure 1*A*. Tubules are either empty or filled with debris probably from sloughed germinal cells. PAS × 74.

(table 4); histologically there was a general reduction in tubule size and in height and compactness of the germinal epithelium (fig. 1 *A*). There was also marked reduction in sperm production and much sloughing of germ cells, as reflected in the content of the ducts of the caput epididymis (fig. 1 *B*). After 9 weeks the testes and epididymes of two predepleted rats showed more marked injury than at 6 weeks (fig. 2, *A B*). The testes of a third rat from this group, however, were essentially normal. Testes of the conventional EFA-deficient rats, however, were normal at 9 weeks and the epididymides contained a full complement of sperm (fig. 3, *A* and *B*). After 16 weeks, unilateral orchiectomy revealed that testes of the conventional EFA-deficient rats were still normal, but at 25 weeks the remaining testes showed marked degeneration (data not shown).

Total lipid and phospholipid contents of both types of EFA-deficient testes were within the normal range (table 4). The fatty acid patterns at 6 weeks showed a greater degree of EFA deficiency in the predepleted than in the conventionally depleted testes, as evidenced by slightly higher percentages of the ω9 fatty acids and lower percentages of the ω6 fatty acids. Similar relationships were true also at 9 weeks although the magnitude of changes between 6 and 9 weeks were small.

Thin-layer chromatographic separation of the phospholipids (table 5) did not reveal any differences between the predepleted or conventional testes at 6 weeks. At 9 weeks, the former tissues had slightly less phosphatidyl choline and more phosphatidyl ethanolamine but the differences were small. Comparison of the testes of EFA-deficient rats with those from normal rats ingesting corn oil indicates that the relative distribution of phosphatide classes is not altered in the early stages of EFA deficiency.

Fatty acid analyses of the two predominant phosphatides, phosphatidyl choline and phosphatidyl ethanolamine, after 9

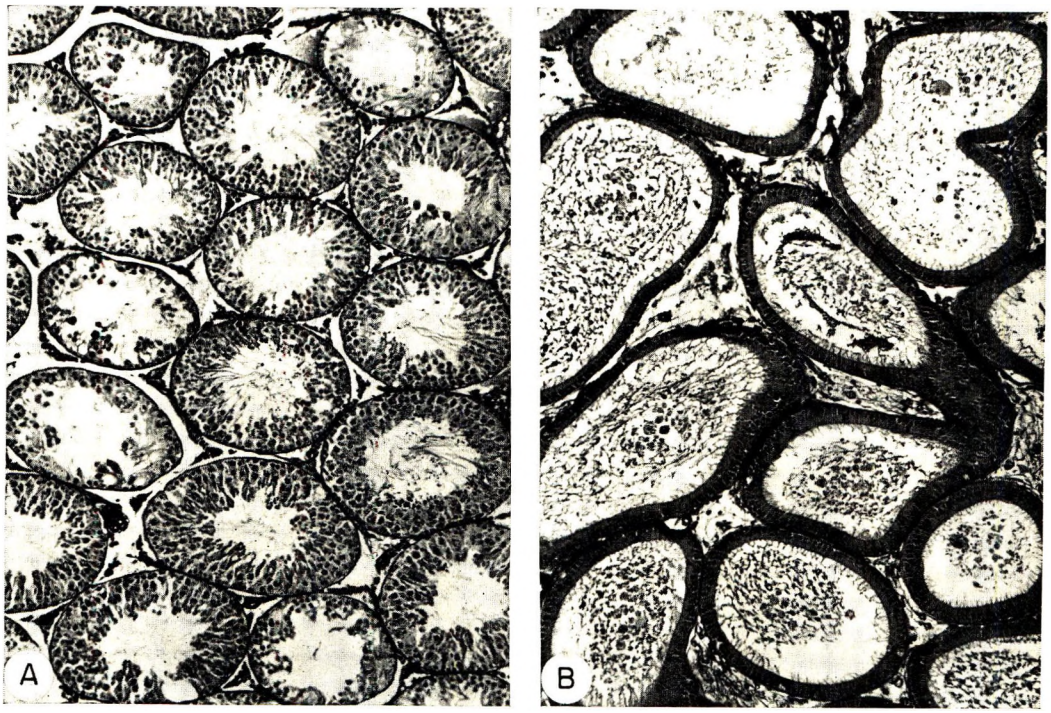

Fig. 2(*A*) Testis from rat predepleted of EFA. Tissue removed 9 weeks after weaning. Loss of germinal epithelium is marked in many tubules. PAS × 74. (*B*) Caput epididymis from testis in figure 2 *A*. Tubular contents are sparse with clumped sperm and cellular debris. PAS × 74.

weeks of EFA deficiency are shown in table 6. The differences between the predepleted and conventional EFA-deficient rats were similar to those reflected in the total lipid fatty acids in table 4. In the predepleted testes, the percentages of ω6 fatty acids were slightly lower in both phosphatides, whereas the percentages of ω9 acids were higher.

DISCUSSION

These experiments emphasize the significance of the preweaning diet on the eventual condition of the testes in EFA deficiency. When young rats obtained from a commercial breeder [11] were allowed access to their mother's stock diet during the lactation period, the subsequent development of EFA deficiency symptoms, such as scaliness of tails and feet, reduced growth rate and increased water intake, appeared in the usual time of about 10 to 12 weeks, yet the testes showed no more than mild degrees of injury after 26 weeks. In contrast, offspring from the NIH Production Section when given access to only an EFA-deficient diet during the last 11 days of lactation and thereafter, showed symptoms of EFA deficiency and evidence of testicular injury — histologically and grossly — within 6 weeks after weaning.

The considerable difference in time required to obtain testicular damage in conventionally depleted rats from a commercial source as compared with rats from our own production unit is probably due either to a higher content of EFA in the stock diet of the former producer or to longer exposure of the young rats to stock diet while in transit for 1 to 2 days.

Predepletion of rats for EFA-deficiency studies is rarely used. Hauge and Nicolaysen (13) reported accelerated deficiency symptoms in rats whose mothers were fed the EFA-deficient diet early in pregnancy. Holman and Peifer (14) found that 15-day-old rats developed dermal symptoms faster than did 21-day-old rats. The study

[11] See footnote 2.

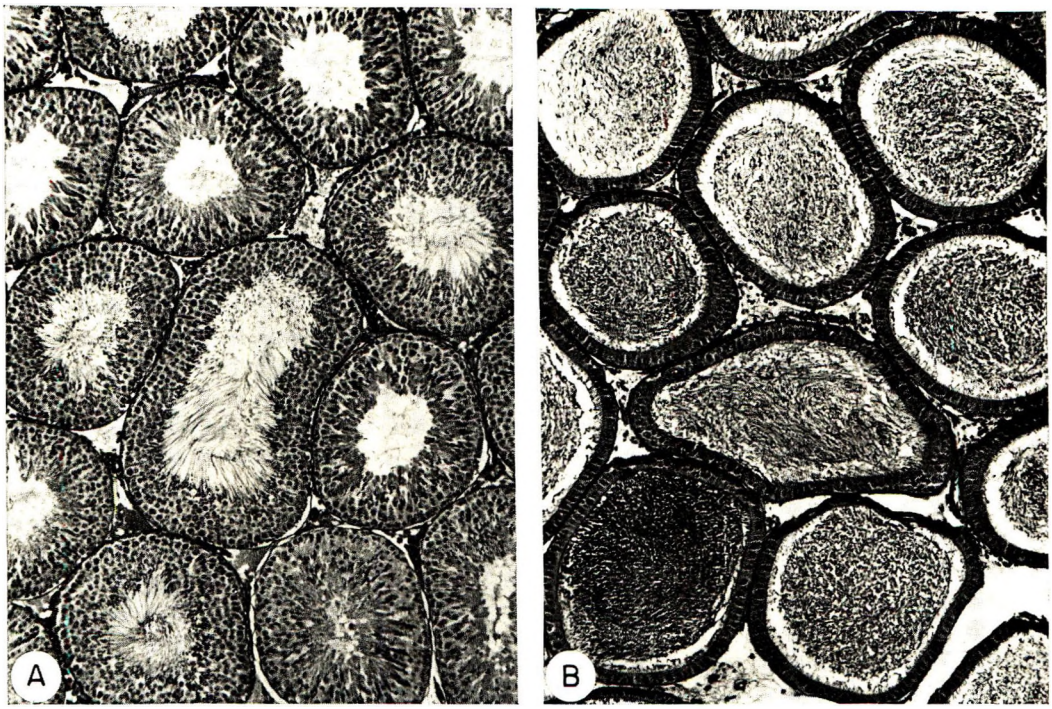

Fig. 3 (*A*) Testis from rat conventionally depleted of EFA. Tissue removed 9 weeks after weaning. Compare tubules with those in figure 2 *A*. PAS × 74. (*B*) Caput epididymis from testis in figure 3 *A*. Compare with those in figure 2 *B*. PAS × 74.

was prompted by their observation that similar lots of 21-day-old rats varied considerably in the time required for EFA deficiency to develop. These investigators also found that feeding 1% cholesterol with 1% hydrogenated coconut oil to 21-day-old rats accelerated EFA-deficiency symptoms, including smaller testes by week 5.

In one experiment (not reported) with 21-day-old weanling rats from a commercial source, feeding 1% cholesterol with 3% hydrogenated coconut oil did not result in abnormal testes by 16 weeks, even though dermal symptoms were marked. It would appear that the predepletion technique may be more effective than the feeding of cholesterol for accelerating EFA-deficiency effects on the testes. Addition of 3% hydrogenated coconut oil or 3% trilaurin without cholesterol (exp. 2) did not have any accelerating effect over the fat-free diet alone. Aaes-Jørgensen et al. (6), however, successfully used 28% saturated fat to increase EFA deficiency.

No correlation was observed between any aspect of lipid metabolism and the onset of testicular degeneration in EFA deficiency. As shown in experiments 1 and 4, by weeks 6 or 7 the total fatty acid pattern is essentially fixed and only slight changes occur over as long a period as 34 weeks. Yet, it is possible for the testis to remain histologically normal for as long as 26 weeks (exp. 2, table 3) with no marked change in lipid composition. When a comparison is made of the testis fatty acids of predepleted and conventionally depleted rats (tables 4 and 6), in general the ω9 acids are slightly higher and the ω6 acids lower in the predepleted animal. However, *within either group of rats at any time period the fatty acid patterns of testes showing histological damage were not different from testes which were histologically normal.* Similarly, examination of the values for total lipid, total phospholipid and cholesterol (where performed) does not show any differences which would suggest that any one lipid component is more

TABLE 5

Phospholipid composition of testes from conventional or predepleted essential fatty acid deficient rats [1]

	Predepleted EFA deficient		Conventional EFA deficient		3% corn oil controls	
No. of weeks	6	9	6	9	6	9
No. of rats	3	3	3	3	2	2
Lysophosphatidyl choline	1.6 ± 0.3	2.1 ± 0.2	2.1 ± 0.7	1.8 ± 0.1	1.6 ± 0.2	1.8 ± 0.9
Sphingomyelin	7.6 ± 0.7	7.5 ± 0.7	7.9 ± 0.9	6.7 ± 0.2	6.6 ± 0.4	6.4 ± 0.8
Phosphatidyl choline	44.0 ± 1.7	42.4 ± 3.1	43.4 ± 1.1	45.3 ± 0.9	42.2 ± 1.2	46.3 ± 1.3
Phosphatidyl inositol + phosphatidyl serine [2]	11.9 ± 0.7	11.4 ± 0.6	12.0 ± 0.0	12.6 ± 0.2	12.0 ± 0.6	11.4 ± 0.8
Phosphatidyl ethanolamine	28.1 ± 1.4	30.9 ± 1.2	27.8 ± 0.7	27.4 ± 0.3	30.4 ± 1.0	27.4 ± 0.3
Neutral lipid [3]	6.9 ± 0.3	5.8 ± 0.1	6.8 ± 0.2	6.0 ± 0.6	7.3 ± 0.3	6.6 ± 0.6

[1] See footnotes 1, 2 and 3, table 4 for dietary treatments. Values are percentage of total phosphorus; means ± SE. Average recovery of phosphorus applied to plates was 94.7 ± 2.7%.
[2] These areas were combined because of incomplete separation.
[3] Material at the top of the thin-layer plates.

TABLE 6

Fatty acid composition of choline and ethanolamine phosphatides of testes from conventional or predepleted essential fatty acid-deficient rats [1]

	Phosphatidyl choline			Phosphatidyl ethanolamine		
	Predepleted EFA deficient	Conventional EFA deficient	3% corn oil controls	Predepleted EFA deficient	Conventional EFA deficient	3% corn oil controls
No. of rats	3	3	2	3	3	2
Fatty acids [2]						
$C_{14:0}$	0.7 ± 0.1	0.5 ± 0.2	—	0.7 ± 0.2	1.5 ± 0.4	—
C_{16} ald	0.5 ± 0.1	0.5 ± 0.1	1.5 ± 0.3	19.1 ± 2.0	17.6 ± 1.6	19.2 ± 1.8
$C_{16:0}$	43.1 ± 3.7	42.0 ± 3.3	40.5 ± 1.9	22.8 ± 1.0	28.0 ± 1.5	23.0 ± 2.2
$C_{16:1}$	1.5 ± 0.2	1.3 ± 0.4	—	—	—	—
C_{18} ald	—	—	—	2.4 ± 0.3	2.0 ± 0.2	3.4 ± 0.6
$C_{18:0}$	6.4 ± 0.4	8.0 ± 0.6	6.5 ± 0.4	8.2 ± 0.2	6.9 ± 0.3	7.1 ± 0.2
$C_{18:1}$	30.5 ± 2.0	26.7 ± 2.8	18.2 ± 1.4	11.1 ± 1.0	10.5 ± 0.3	5.0 ± 0.3
$C_{18:2}$	0.4 ± 0.0	0.5 ± 0.1	3.0 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	1.0 ± 0.1
$C_{20:3\omega9}$	7.7 ± 1.1	6.6 ± 0.4	—	9.4 ± 0.5	6.3 ± 0.4	—
$C_{20:4\omega6}$	3.9 ± 0.6	4.7 ± 0.3	14.3 ± 0.5	8.9 ± 0.6	8.9 ± 0.4	18.1 ± 1.4
$C_{22:3\omega9}$	0.7 ± 0.2	0.6 ± 0.3	—	2.9 ± 0.2	2.6 ± 0.3	—
$C_{22:4\omega9}$	0.9 ± 0.3	0.4 ± 0.3	—	3.7 ± 1.0	2.8 ± 0.3	—
$C_{22:4\omega6}$	—	—	0.8 ± 0.0	—	—	2.3 ± 0.2
$C_{22:5\omega6}$	3.4 ± 0.6	5.7 ± 0.3	13.9 ± 0.7	8.3 ± 0.3	10.1 ± 0.6	19.5 ± 1.7
$C_{22:6\omega3}$	—	—	—	1.1 ± 0.2	1.1 ± 0.3	—

[1] See footnotes 1, 2 and 3, table 4 for dietary treatments. Experimental period was 9 weeks.
[2] See footnote 4, table 1.

critical than others for maintenance of testicular structure and function. Possibly, one or more of the minor phosphatides not analyzed may be the limiting factor in EFA deficiency.

The marked increase in content of $C_{22:5\omega6}$ fatty acid as the rat testis matures (15, 16) has led to suggestions that this acid may have a key biochemical role in spermatogenesis. Our results indicate that the fatty acids of the linoleic acid series are essential for testis development but they are not required for maintenance of the normal testicular architecture and spermatogenesis over a long period. In one experiment (data not reported), three rats

deficient in EFA for 11 weeks were successfully mated with stock females which after 2 weeks had viable fetuses. The testes of the males when analyzed immediately after mating were normal histologically but had fatty acid patterns characteristic of prolonged EFA deficiency. The eventual degeneration of the fully matured EFA-deficient testis may be due to a secondary insufficiency of pituitary gonadotropins (17–19), but conclusive evidence for this has not been provided (17). Examination of pituitaries from several of our rats after 34 weeks of EFA deficiency showed histological changes similar to those previously described (18, 19). Further work is required to clarify the role of essential fatty acids in testicular development and maintenance.

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Protein Quality of Opaque-2 Corn Evaluation in Rats [1,2]

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ABSTRACT A study was made on the protein quality of opaque-2 corn as tested in rats. Opaque-2 corn showed a protein efficiency ratio (PER) of 2.79 as compared with 2.88 for casein at comparable levels of protein in the diet. The results also indicate that processing the corn into corn-masa or tortilla does not alter its high protein quality, although slightly lower PER values were found. Amino acid-supplementation studies indicated that lysine is the first limiting amino acid in opaque-2 corn when it provides less than 10% protein in the diet. Results are presented on the significant improvement obtained when common corn is supplemented with lysine and tryptophan, added together. Evidence that niacin from opaque-2 corn is available to the niacin-depleted rat, as opposed to its low availability for rats in the same condition in common corn, is also provided. Furthermore, the data show that opaque-2 corn has more niacin than common corn. Because of the high quality of opaque-2 corn proteins, efforts should be made to introduce the gene causing high lysine content into local varieties, especially in areas where corn is the most important staple food.

It has been known since 1914 that the quality of corn proteins is poor because they are deficient in the essential amino acids, lysine, and tryptophan (1). The reason behind these deficiencies is that zein, the corn protein which is soluble in alcohol, contributes up to 50% of the total protein in the corn kernel (2–5) in most corn varieties. Zein has been studied extensively, and results have indicated that it contains very low levels of lysine and tryptophan (6–8). Numerous studies (3, 4, 9–12) on the factors affecting the protein quality of corn indicated that both environment and variety had, in several cases, a significant effect on lysine content. It has also been shown that fertilization increases protein content and decreases protein quality. This is due to a total increase in the zein fraction which causes protein quality to decrease (2, 10, 13, 14). None of these studies had indicated the possibilities of increasing the nutritive quality of corn proteins, until Mertz et al. (15) showed that the opaque-2 gene of corn caused a genetic increase in lysine concentration. The studies by Mertz and co-workers (15) indicated also that the lysine increase in opaque-2 corn was the result of change in the distribution of endosperm proteins; opaque-2 corn contains only approximately 22% zein. Chemical analysis of corn protein for amino acids showed that opaque-2 corn contains 4.2 g lysine/100 g of protein, as compared with 2.8 g in regular corn. These results were corroborated by several workers (16–20), and by biological trials carried out with rats by Mertz et al. (17). Because corn constitutes the main staple of many population groups in which protein deficiencies are prevalent, it was of interest to study the nutritional quality of opaque-2 corn more extensively. Furthermore, in several countries in Latin America, corn is consumed in the form of tortillas. Therefore, it is important to learn whether or not the process of tortilla preparation affects the quality of opaque-2 corn protein. This paper presents the results of these studies.

MATERIAL AND METHODS

The opaque-2 corn [3] used in all the experiments was obtained from the United States. It was shipped by air and stored at INCAP laboratories until used.

Representative samples were analyzed for proximate chemical composition by AOAC official methods (21), and for ly-

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[3] Obtained from Purdue University, Lafayette, Indiana.

sine and tryptophan by microbiological assay (22). Niacin content was determined also, by microbiological assay for which *Leuconostoc mesenteroides* with Difco media was used (23).

Tortilla preparation. The method used was previously described by Bressani and Scrimshaw (22) and Bressani et al. (24). The raw corn was first washed with water to remove foreign matter. To 12.5 kg of corn, 24 liters of water and 200 g of lime were added. The mixture was cooked for 90 minutes. It was allowed to cool, the cooking liquor was discarded and the cooked kernels were washed four or five times with water, or until the washings were clear. During this process, the kernels were scrubbed to remove the seed coat which breaks down during the alkaline cooking. The cooked and peeled corn was then ground to a fine masa. Part of the masa was dried with hot air at 80° and part was used for tortilla preparation. The tortillas were dried also by hot air at 80°. The procedure described was applied to both the opaque-2 corn and to a common Guatemalan highland corn. Both the masa and the tortillas were analyzed for lysine and tryptophan content as indicated before, and for niacin content by microbiological methods (22).

Tests with rats. Several studies were carried out, using groups of 12 weanling white rats of the Wistar strain from the INCAP colony. Each group was made up of six male and six female rats, and care was taken that the initial weight was the same between groups within each experiment. The animals were placed in individual all-wire screen cages with raised-screen bottoms. The diets, as well as water, were provided ad libitum for a 28-day period. Changes in weight and amount of food consumed were measured every 7 days.

In the first study, all animals were killed, and blood and liver were collected for total serum protein analysis (25) and for determination of the chemical composition of the liver.

Table 1 describes the composition of the basal diet used in the study for the evaluation of the protein quality of corn made into tortillas, as well as the composition of the basal diet used in all amino acid-supplementation experiments. Total nitrogen was determined in the diets by the macro-Kjeldahl method, for the calculation of protein efficiency, defined as weight gain per gram of protein consumed. The amounts of amino acids added to the basal diet are given in each section, under Results. Their weight replaced an equal weight of corn starch although no corrections were made for the small amounts of nitrogen contributed by their addition.

Physiological availability of niacin. For these studies, 50 rats were given the niacin-depletion diet described by Kodicek and Silson (26) for 35 days, as shown in table 1. After this period, the surviving animals were distributed by weight into eight groups; they were fed the basal diet in which the corn was replaced by different corn preparations, as described under Results. The diets made with raw or cooked common corn contained an average of 8.2% protein, those from raw and cooked opaque-2 corn contained 8.9% protein.

TABLE 1

Composition of basal diets used in feeding tests

	Diet			
	1 [1]	2 [1]	3 [2]	4 [3]
Corn	90.0	—	72.0	40.0
Casein (vitamin free)	—	11.0	—	3.5
Mineral mixture [4]	4.0	4.0	4.0	3.0
Cottonseed oil	5.0	5.0	5.0	2.0
Cod liver oil	1.0	1.0	1.0	—
L-Cystine	—	—	—	0.1
Sucrose	—	—	—	51.4
Corn starch	—	79.0	18.0	—
Total	100.0	100.0	100.0	100.0
Vitamin solution, ml [5]	4	4	4	4 [6]

[1] Diets 1 and 2 were used for PER assay of corn preparations. Three diets were prepared from opaque-2 corn and three from common corn, in which the amount of raw corn was replaced by an equal quantity of corn-masa and tortilla flour.

[2] Diet 3 was used for amino acid supplementation studies. Opaque-2 corn was always tested in the amount shown. The level was raised to 82% for the common corn because of its lower protein content. The amino acids added replaced an equal weight of corn starch. The amounts are indicated under Results.

[3] Diet 4 was fed for 35 days to cause niacin depletion in the rats. For the availability study, the common corn was replaced by different preparations as indicated under Results.

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[6] Same vitamin solution (Manna and Hauge), but without added niacin.

The second part of the experiment was carried out for a 28-day period, and the rats were allowed to feed and drink water ad libitum. As before, the animals were placed in individual all-wire screen cages with raised-screen bottoms, and weight change and food consumed were recorded every 7 days.

RESULTS

The nitrogen, lysine, tryptophan and niacin content of opaque-2 corn and the common corn used in the study, as well as the content of the above nutrients in the respective corn-masa and tortilla, are shown in table 2. As indicated, opaque-2 corn contained higher amounts of all these nutrients. Table 3 shows the results obtained when common and opaque-2 corn, raw or processed into masa and tortillas, were tested in rats. Weight gain was essentially the same for the three preparations from the common corn. Slightly lower values were obtained from opaque-2 corn made into masa and tortilla than those obtained with raw corn. In all cases, growth of the rats fed raw opaque-2 corn and the two preparations was four to five times greater than the corresponding response from rats fed common corn. Food intake was similar within the same corn preparations, but it was about twice as high for the animals fed opaque-2 corn as compared with common corn. The PER values for the masa and tortilla made from common corn were slightly higher than the value obtained for the raw corn. The PER value of the tortilla made from opaque-2 corn, however, was slightly lower than the value observed for the raw corn and the masa. The differences

TABLE 2

Lysine, tryptophan and niacin content of opaque-2 and common corn and preparations

Corn	Nitrogen	Lysine	Tryptophan	Niacin
	%	*g/16 g N*	*g/16 g N*	*mg/100 g corn*
Opaque-2				
Raw	1.64	4.5	1.5	2.20
Masa	1.67	4.6	1.5	2.47
Tortilla	1.64	4.2	1.5	2.01
Common				
Raw	1.16	3.6	0.62	1.62
Masa	1.12	3.1	0.57	1.63
Tortilla	1.06	3.1	0.49	1.87

TABLE 3

Average weight gain, food intake and protein efficiency ratio of different preparations from opaque-2 and common Guatemalan corn and their effect on total serum proteins, liver weight, fat and protein contents

Diet fed	Avg wt gain [1]	Food intake	PER	Total serum proteins	Liver		
					Fresh wt	Fat [2]	Protein [3]
	g			*g/100 ml*	*g*	%	%
Common Guatemalan corn							
Raw	25 ± 1.9 [4]	248 ± 11.5 [4]	1.49 ± 0.07 [4]	5.58	3.42	31.7	48.0
Masa	28 ± 2.0	240 ± 10.6	1.66 ± 0.05	5.02	3.18	28.0	49.8
Tortilla	27 ± 1.7	238 ± 8.8	1.55 ± 0.07	5.24	3.42	29.3	49.5
Opaque-2 corn							
Raw	130 ± 6.2	455 ± 12.7	2.79 ± 0.07	5.50	7.36	21.1	47.6
Masa	115 ± 2.9	389 ± 8.3	2.76 ± 0.04	4.89	6.31	18.2	47.9
Tortilla	115 ± 5.2	414 ± 14.3	2.66 ± 0.04	6.47	6.40	20.2	48.2
Casein	132 ± 5.9	408 ± 10.7	2.88 ± 0.06	6.31	7.81	11.6	46.2

[1] Average initial weight: 47 g.
[2] Dry weight basis.
[3] Dry weight basis, fat free.
[4] SE.

were not significant. As with growth, the PER values for the raw opaque-2 corn and preparations were significantly higher (1%) than the respective values for the common corn. The performance of the rats fed the raw opaque-2 corn was only slightly lower than the values observed for the rats fed the casein diet.

Table 3 also summarizes the values for serum proteins and liver composition. Total serum proteins were similar in rats fed either corn, although the rats fed tortilla from opaque-2 corn gave higher values. Fresh liver weight was twice as high in the rats fed opaque-2 corn and its preparations than the values obtained from rats fed common corn and its preparations. Liver fat was lower for the animals fed opaque-2 corn than for those fed common corn, but higher than for the casein control. Liver protein as a percentage was similar for all groups.

Table 4 shows the results of amino acid supplementation of common corn and opaque-2 corn. With respect to opaque-2 corn, a small response was obtained from lysine supplementation, which was not altered when tryptophan or tryptophan and isoleucine were added with lysine. For the common corn, no response was obtained from lysine addition alone. A significant effect was observed, however, when both lysine and tryptophan were added. Isoleucine had only a slight effect which was not significant. Table 5 presents the results of further studies. The addition of tryptophan alone to both opaque-2 corn and to the common corn did not change weight gain

TABLE 4

Effect of amino acid supplementation on opaque-2 and common corn

Amino acid supplement	Avg wt gain [1]	PER
	g	
Opaque-2		
None	90 ± 3.5 [2]	2.72 ± 0.05 [2]
+ 0.40% L-lysine·HCl	96 ± 5.8	2.94 ± 0.07
+ 0.40% L-lysine·HCl + 0.10% DL-tryptophan	91 ± 4.6	2.82 ± 0.07
+ 0.40% L-lysine·HCl + 0.10% DL-tryptophan + 0.20% DL-isoleucine	94 ± 5.2	2.96 ± 0.08
Common corn		
None	38 ± 2.6	1.47 ± 0.08
+ 0.40% L-lysine·HCl	28 ± 4.0	1.41 ± 0.18
+ 0.40% L-lysine·HCl + 0.10% DL-tryptophan	75 ± 5.5	2.47 ± 0.08
+ 0.40% L-lysine·HCl + 0.10% DL-tryptophan + 0.20% DL-isoleucine	77 ± 4.9	2.53 ± 0.09

[1] Average initial weight: 47 g.
[2] SE.

TABLE 5

Effect of supplementing opaque-2 and common corn with tryptophan alone and with other amino acids

Amino acid supplement	Avg wt gain [1]	PER
	g	
Opaque-2		
None	67 ± 4.6 [2]	2.60 ± 0.08 [2]
+ 0.10% DL-tryptophan	67 ± 4.6	2.66 ± 0.09
+ 0.10% DL-tryptophan + 0.40% L-lysine·HCl + 0.15% DL-methionine	72 ± 5.5	2.81 ± 0.10
Common corn		
None	27 ± 2.3	1.13 ± 0.11
+ 0.10% DL-tryptophan	26 ± 2.0	1.16 ± 0.09
+ 0.10% DL-tryptophan + 0.40% L-lysine·HCl + 0.15% DL-methionine	60 ± 5.5	2.18 ± 0.16

[1] Average initial weight: 48 g.
[2] SE.

or PER. The simultaneous addition of lysine and of methionine with tryptophan, however, improved both weight gain and PER, particularly in the case of common corn. In all cases, the values obtained from the opaque-2 corn were higher than the values obtained from common corn. In this experiment food intake was lower than in other studies, which could account for the lower weight gains obtained.

Table 6 presents additional results for the amino acid supplementation of opaque-2 corn. Again, it appears that lysine addition, alone or in the presence of tryptophan and of tryptophan and methionine, increased weight gain and PER. Methionine added alone did not have any effect. The lower section of table 6 shows results of further tests, indicating no effect from the addition of threonine or methionine alone. Lysine added in the presence of tryptophan and threonine resulted in a significant increase in PER, but not in weight gain.

Finally, table 7 summarizes the results obtained in the studies carried out to determine the availability of niacin from opaque-2 corn. It can be seen that the animals fed the raw common corn diet lost weight without niacin supplementation, but the addition of the vitamin resulted in a significant increase in weight gain and reduced mortality. On the other hand, feeding the raw opaque-2 corn diet with and without niacin addition resulted in a similar weight gain for both cases, and also for the diet made from common corn supplemented with niacin. Lime cooking of the common corn apparently caused a beneficial effect, since the animals maintained their weight. Lime cooking of the opaque-2 corn reduced weight gain when compared with untreated corn. When both

TABLE 6

Amino acid supplementation of opaque-2 corn

Amino acid supplement	Avg wt gain	PER
	g	
None	86 ± 4.0 [1]	2.45 ± 0.06 [1]
+ 0.05% L-lysine·HCl	89 ± 4.9	2.65 ± 0.06
+ 0.10% L-lysine·HCl	92 ± 5.8	2.78 ± 0.06
+ 0.10% DL-methionine	83 ± 4.3	2.59 ± 0.07
+ 0.10% lysine·HCl + 0.05% DL-tryptophan	83 ± 3.8	2.70 ± 0.07
+ 0.10% lysine·HCl + 0.05% DL-tryptophan + 0.10% DL-methionine	90 ± 4.3	2.85 ± 0.05
Average initial weight: 50 g		
None	77 ± 3.5 [1]	2.78 ± 0.05 [1]
+ 0.20% DL-threonine	77 ± 4.6	2.74 ± 0.06
+ 0.15% DL-methionine	84 ± 4.0	2.51 ± 0.06
+ 0.40% L-lysine + 0.10% DL-tryptophan + 0.20% DL-threonine	69 ± 4.9	2.98 ± 0.11
Average initial weight: 46 g		

[1] SE.

TABLE 7

Niacin and amino acid supplementation of raw and lime-treated opaque-2 and common corn

Treatment	Avg wt gain [1]	Mortality
	g	
Common corn raw	−9	3/4
Common corn raw + niacin [2]	55	1/4
Opaque-2 raw corn	52	1/4
Opaque-2 raw corn + niacin [2]	56	0/4
Lime-treated common corn (masa)	1	3/4
Lime-treated opaque-2 corn (masa)	36	1/4
Common raw corn + lysine + tryptophan + niacin [2]	54	0/4
Opaque-2 corn + lysine + tryptophan + niacin [2]	69	0/4

[1] Average initial weight: 46 g.
[2] Levels added in milligrams per 100 g of diet: L-lysine·HCl, 100; DL-tryptophan, 50; and niacin, 4.

corn samples were supplemented with lysine, tryptophan and niacin, growth response increased for the opaque-2 corn but not for common corn, comparing these results with those of the niacin-supplemented corns. When the comparison is made with the results from unsupplemented corns, however, the amino acids and niacin improved the nutritive value of common corn.

DISCUSSION

The results of the present investigation corroborate the findings of Mertz et al. (15,17), indicating that the opaque-2 corn has a high protein quality. As shown by the same authors, and likewise by data presented in this paper, the high quality of opaque-2 corn protein is due to its higher content of lysine and tryptophan, in comparison with common corn. Some of the differences in weight gain and PER could be attributed also to the higher levels of total protein in the diets made from opaque-2 corn. These diets contained an average of 10.4% protein whereas those from common corn contained only 6.9%. The difference in protein content, however, probably affected weight gain more than PER, since it has been shown (10) that low protein-containing corn samples have a better protein quality than corns with a high protein content. In other studies (27) common Guatemalan corns with a higher protein content than the one used in the present study gave a PER value of around 1.0.

The results indicate further that lime cooking of opaque-2 corn for the preparation of tortilla causes only a small, not significant, decrease in its protein quality. The small decrease could be due to the inactivation of some of the lysine, since the process of tortilla preparation requires the use of high temperatures (22,24) which are known to make certain amino acids, particularly lysine, less available to the organism (24).

Studies carried out by Rogler (28) have indicated that the high quality of the protein of opaque-2 corn cannot be shown when it is used in combination with protein concentrates, as tested in chicks. Similar results were found by Bressani (29), when testing the use of opaque-2 corn in vegetable protein mixtures with rats. Therefore, it is of interest to learn the reason behind these findings. Because the response is probably related to amino acid balance, studies were carried out to determine the limiting amino acids in opaque-2 corn protein. The results of the present study show that neither methionine, tryptophan nor threonine are first limiting amino acids, since, when they were added alone, no response was evident. Similar results were reported by Clark (30) in man. Rogler (28) reported that opaque-2 corn protein responded to methionine supplementation when used with soybean meal in chick nutrition studies.

Examination of all data obtained when opaque-2 corn was supplemented with lysine, added alone or with other amino acids, showed that the first limiting amino acid is lysine. Statistical analysis of the difference between all PER data from the control groups compared with the PER data of all groups supplemented with lysine, or groups containing lysine together with other amino acids, was highly significant. There was no statistically significant difference in PER between the lysine-supplemented groups and groups fed opaque-2 corn supplemented with lysine plus other amino acids. Apparently, although the content of this amino acid is higher in opaque-2 corn than in common corn, its concentration in the former is not high enough to supply all the lysine needed by the rat at the 9% protein level in the diet. That the corn is deficient can also be deduced from observation of the fat in the liver, which, although lower than liver fat when common corn is fed, was still higher than the fat found in the liver of rats fed casein. Further evidence can be inferred from the studies in which opaque-2 corn was used in three vegetable protein mixtures, one based on cottonseed, one on soybean, and the third on equal parts of both proteins. Opaque-2 corn and cottonseed protein gave a higher index of protein value than common corn and cottonseed protein. Similar results were obtained when opaque-2 corn was tested with cottonseed and soybean proteins, but not when mixed with soybean alone (29). It is well known that cottonseed is deficient in lysine whereas soybean is a rich source of this amino acid (31). It is recognized, however, that the lysine

deficiency is of academic interest. Awareness of this limitation is of value in the use of this corn in diet formulation and in practical applications.

The results on the availability of niacin from opaque-2 corn are of interest. They indicate that the niacin from opaque-2 corn is available to the rat, as opposed to that from common corn, although it is true that opaque-2 corn contained more niacin than common corn. Higher concentration, however, does not necessarily mean more availability, since there are corn samples which contain as much niacin as the opaque-2 corn used in the present study. However, the lime treatment to which corn is subjected when tortilla is made causes a loss of the vitamin, shown previously by Bressani and Scrimshaw (22) and Bressani et al. (24), which could explain the lower weight gain of the animals fed cooked opaque-2 corn. The data also indicate that the theory on the low availability of niacin from corn should be revised (26,32,33). It appears that amino acid balance and tryptophan deficiency are more fitting, and that lime treatment in regular corn causes an increase in tryptophan availability rather than a release of bound niacin. Furthermore, the results showed that common corn supplemented with niacin produced as good a weight gain as the group fed common corn supplemented with niacin, lysine and tryptophan. This would mean that lysine and tryptophan added to common corn in the presence of niacin did not have any additional effect. It must be considered, however, that the basal corn diet contained 3.5% casein, which provided, together with corn, an amino acid pattern which was different from that of corn alone. The loss of animals even after the application of the different treatments, with the exception of the first group, was probably due to the condition of extreme niacin depletion which the animal was in at the beginning of the study.

From the practical point of view, great efforts should be made to introduce corn varieties with the opaque-2 gene, particularly in areas of the world where corn is the main staple food.

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Whole-body Retention of Strontium-85 in Swine Given Sodium Alginate or Barium and Sodium Sulfates [1]

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ABSTRACT Dry sodium alginate was given orally to immature and mature swine 10 minutes following oral administration of strontium-85. The whole-body retention of radiostrontium was higher in treated pigs, but was not statistically different from that of control animals. The ineffectiveness of sodium alginate may be attributable to a tough coating of alginic acid which is reported to form around dry sodium alginate in dilute acid, presumably making the alginate unavailable for metal binding. A barium sulfate and sodium sulfate mixture, given orally to pigs 10 minutes following oral administration of strontium-85, significantly decreased whole-body retention of radiostrontium in young swine; on day 1, only in older pigs. Whole-body strontium-85 retention in young pigs given the sulfate mixture was significantly less than that of control swine through day 10 following oral radioactivity dosing. Differences in the whole-body retention of strontium-85 on day 3 and day 10 following oral administration of the radionuclide were not significant in treated and control mature swine, indicating that treatment need not be repeated in mature swine, but might be warranted in young animals.

One of the most important fission products produced in large amounts by nuclear explosions and reactors is strontium-90. It is absorbed from the gastrointestinal tract and deposited principally in the skeleton. Slow turnover of strontium-90 in bone is a source of continuing radiation exposure to the bone and contiguous structures.

Selective inhibition of radiostrontium absorption from the gastrointestinal tract was demonstrated in rats and man by oral administration of sodium alginate (1–9). Several other investigators, however, found sodium alginate ineffective in rats and young chickens in decreasing gastrointestinal absorption of radiostrontium (10–12). In addition, reduced absorption of radiostrontium from the gastrointestinal tract was observed in rats by oral administration of barium and sodium sulfates (10, 13, 14).

This study was designed to investigate the therapeutic effect of sodium alginate as well as barium and sodium sulfates on gastrointestinal absorption of strontium-85 in swine. Strontium-85 rather than strontium-90 was utilized because it has a physical half-life of 65 days, it is a gamma emitter, and it is easily measured in vivo by a whole-body radiation counter.

MATERIALS AND METHODS

Experimental animals. Sixteen swine were used in this experiment: eight Yorkshire-Hampshire crosses, about 1 year old, weighing 135 to 205 kg; and eight Hampshires, 3.5 months old, weighing about 45 kg.

Radioactivity. Approximately 0.25 μCi of strontium-85 was placed in a gelatin capsule, dried under a heat lamp and placed within a larger starch-filled capsule. A capsule was administered to each pig via a balling gun.

Substances tested. Sodium alginate,[3] barium sulfate (USP) and sodium sulfate (NF) were the substances employed in an attempt to reduce gastrointestinal absorption of orally administered strontium-85.

A mixture of 4 mM barium sulfate and 4 mM sodium sulfate, prepared in an aqueous solution, was administered to three pigs of each group. Each mature pig received 400 ml of this solution; and each

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TABLE 1

Fractional retention of radiostrontium in control swine and swine treated with sodium alginate and barium and sodium sulfates

Day no.	Sodium alginate				Barium and sodium sulfate				Control	
					Age 3.5 months					
Swine no.	A-1	A-2	A-3		B-1	B-2	B-3		C-1	C-2
1	1.07 [1]	0.82 [1]	1.04 [1]	0.97 ± 0.13 [2]	0.34 [1]	0.18 [1]	0.69 [1]	** 0.50 ± 0.18 [2]	1.01 [1]	0.90 [1]	0.95 ± 0.08 [2]
2	0.84	0.43	0.49	0.59 ± 0.22	0.08	0.11	0.28	** 0.16 ± 0.10	0.59	0.32	0.45 ± 0.16
3	0.64	0.35	0.28	0.42 ± 0.19	0.06	0.10	0.18	** 0.11 ± 0.08	0.40	0.19	0.29 ± 0.15
10	0.54	0.30	0.22	0.35 ± 0.16	0.09	0.11	0.16	** 0.12 ± 0.04	0.38	0.19	0.28 ± 0.13
					Age 1 yr					
Swine no.	1	2	3		4	5	6		7	8	
1	0.99	0.69	0.93	0.87 ± 0.17	0.51	0.36	0.20	* 0.39 ± 0.17	0.79	0.60	0.70 ± 0.13
2	0.31	0.06	0.16	0.28 ± 0.18	0.07	0.12	0.17	0.12 ± 0.05	0.14	0.11	0.13 ± 0.02
3	0.11	0.03	0.09	0.08 ± 0.05	0.06	0.05	0.11	0.07 ± 0.04	0.07	0.07	0.07 ± 0.00
10	0.07	0.02	0.07	0.05 ± 0.04	0.05	0.04	0.09	0.06 ± 0.03	0.06	0.07	0.07 ± 0.01

[1] Numbers in this column are the relative retentions, R_t, of strontium-85, calculated as in footnote 6 of text.
[2] Mean $R_t \pm$ sp.
* Retentions significantly different from control values ($P < 0.1$).
** Retentions significantly different from control values ($P < 0.05$).

immature pig, 100 ml, by stomach tube within 10 minutes following oral administration of the encapsulated strontium-85. Feed was withheld 24 hours before administration of the sulfates and strontium-85, but the pigs were allowed to drink water ad libitum.

Three pigs of each group were given sodium alginate mixed with their ration following a 24-hour fasting period and oral administration of the encapsulated strontium-85; 454 g sodium alginate was given with 908 g feed [4] in a single ration to the older pigs; 227 g sodium alginate with 454 g feed,[5] to the young pigs.

Two pigs of each group received only a capsule of strontium-85 and served as controls.

Strontium-85 measurement. The Illinois Animal Science Counter (ILLASCO (15)) was used for all measurements of strontium-85. All experimental swine were counted 2 hours following dosing and on days 1, 2, 3 and 10.

Determination of strontium-85 retention. The retention, R_t, of strontium-85 at any time t was calculated according to a modification of the relationship used by Decker et al. (16).[6]

RESULTS

The data of fractional whole-body retention of radiostrontium in treated and control swine are recorded in table 1.

The average values of the retention of strontium-85 by the 3.5-month-old pigs treated with sodium alginate were greater than those for the control animals, but the difference was not statistically significant. The average values of radiostrontium retention in the 3.5-month-old pigs treated with the mixture of barium and sodium sulfates, however, were significantly less than those of the control animals ($P < 0.05$), as determined by the statistical t test for difference between population means (17).

[4] Purina Groweena 2101, Ralston Purina Company, St. Louis, Mo.
[5] See footnote 4.
[6] $R_t = \frac{C_t \times S_0}{C_0 \times S_t}$, where C_0 = measured swine counts per minute, at time of injection, $t = 0$; C_t = measured swine count in counts per minute, at time t in days after injection; S_0 = measured standard count in counts per minute, at time of injection, $t = 0$; and S_t = measured standard count in counts per minute, at time t in days after injection.

In group 2 of swine, 1 year of age, strontium-85 retention by animals treated with sodium alginate or a barium and sodium sulfate mixture did not differ statistically from the control animals, except for the average retention value on day 1 ($P < 0.1$) in pigs given the barium and sodium sulfate mixture.

The age of the swine at the time of treatment with the sulfate mixture appears significant, since only the 3.5-month-old pigs demonstrated a significant difference in strontium-85 retention on day 10 compared with control animals.

DISCUSSION

The whole-body retention of radiostrontium was increased, though not significantly, by treatment with sodium alginate in both 3.5-month-old and 1-year-old swine following oral administration of strontium-85 compared with control animals. The increased values of strontium-85 retention in swine agree with those observed in rats and young chickens by several investigators (10–12). The majority of investigators (1–9) have reported, however, that sodium alginate was highly effective in selectively decreasing absorption of radiostrontium from the gastrointestinal tract of several species.

The ingestion of capsules of dry sodium alginate had no effect on the absorption of either calcium or strontium. A tough coating of alginic acid which forms around dry sodium alginate in dilute acid was presumed to decrease the availability of alginate for metal binding (4). An aqueous solution of sodium alginate with a low degree of viscosity reduced retention of strontium-85 in man by a factor of nine (5).

In the present experiment, the ineffectiveness of sodium alginate in swine in reducing gastrointestinal absorption of strontium-85 may be attributed to the administration of sodium alginate in dry form. Other factors that may be involved are the amount of available binding agents in the alginate preparation, time of the administration of alginate, and palatability associated with viscosity of the product.

The orogastric intubation of a mixture of barium and sodium sulfates 10 minutes following oral administration of strontium-85 resulted in a significant decrease ($P < 0.05$) in the average values of radiostrontium retained by the 3.5-month-old pigs in comparison with that retained by control animals. The whole-body retention of radiostrontium by the 1-year-old swine, following administration of the barium and sodium sulfate mixture, was significantly less ($P < 0.1$) than that by the control pigs only at day 1. From day 2 on, however, retention was the same as for the control pigs, which means that absorption was not impaired at all by the sulfate mixture.

Volf and Roth (18) reported that sodium sulfate can dilute the intestinal contents and stimulate their gastrointestinal passage by inhibiting the absorption of water from the tract. In the presence of strontium ions, the less soluble strontium sulfate is formed. Barium sulfate minimizes the absorption of radiostrontium mainly because of its adsorptive property (18).

In this experiment, whole-body retentions of strontium-85 in swine at day 3 and day 10 were not significantly different in sulfate-treated and control mature pigs. This agrees with results of other investigators (13) who found that the retention of strontium-85 in treated and control groups of rats did not change from day 2 to day 8 after administration, indicating that treatment need not be repeated. Because whole-body retention values in young swine treated with the sulfate mixture through day 10 were significantly different from control animals, however, it is suggested that repetition of the sulfate treatment might be advantageous in reducing the absorption of radiostrontium in young animals. Extrapolation of these findings to the human population might lead to tenuous conclusions. Nevertheless, such treatment might prove beneficial to exposed human beings because of many similarities in the gastrointestinal physiology of man and swine.

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Effect of Varying Dietary Protein-Magnesium Ratios on Nitrogen Utilization and Magnesium Retention in Growing Rats [1]

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ABSTRACT To test the effect of different dietary protein-magnesium ratios on nitrogen and magnesium utilization, male weanling rats were fed diets with 12 or 36% casein and with 0.01, 0.05 or 0.1% magnesium for 5 weeks. Signs of magnesium deficiency developed in rats fed the 0.01% magnesium diets. Food efficiency and net nitrogen utilization were specifically affected by magnesium intake on the high protein diets; the 0.05% magnesium level was apparently not adequate for optimum growth and protein utilization. Other criteria which changed with magnesium intake were plasma proteins and plasma and liver ribonuclease. Total carcass and plasma magnesium concentrations on 0.01% magnesium intakes were slightly lower in animals fed the high protein diet. Magnesium retention and utilization were more efficient, however, with the 36% than the 12% casein diet at all levels of magnesium intake. This latter finding conflicts with previous reports in the literature. Possible reasons for the discrepancy are discussed.

An interdependence of dietary protein and magnesium has been observed in rats (1–4), guinea pigs (5) and chicks (4). In general, when growing or protein-depleted animals were fed an inadequate level of magnesium at either high or low levels of dietary protein, the higher level of protein resulted in reduced weight gains and more severe signs of magnesium deficiency. In all instances cited, the effects of magnesium depletion were less severe with low protein diets. These observations were interpreted to demonstrate a direct relationship between magnesium requirements and protein intake, presumably because of the critical involvements of magnesium in protein synthesis. Bunce et al. (4) reported additional effects of low magnesium intakes on indicators of protein metabolism: significant reduction in plasma proteins and abnormal urinary excretion patterns for amino acids. These authors also found increased urinary losses of magnesium when protein intakes were increased, suggesting a greater turnover of magnesium in animals on high protein intakes.

The present investigation was undertaken, in part, to aid in the selection of some blood parameters to be used as indicators of protein utilization in a metabolic study with adolescent boys on varying intakes of protein and magnesium. One of the parameters chosen was plasma ribonuclease, an enzyme which had been reported by Zigman and Allison (6) and Allison et al. (7, 8) to vary inversely with protein intake and utilization in plasma and livers of rats. If protein synthesis were adversely affected by low magnesium intakes, the level of plasma or tissue ribonuclease might be expected to change in states of absolute or relative magnesium deficiency. For this purpose, it was necessary initially to study the effect of different protein-magnesium ratios on overall nitrogen utilization, in relation to magnesium retention. In general, the results of this investigation confirm and extend previous observations concerning the effect of magnesium intake on protein utilization. The effects of high protein intake on magnesium retention, however, conflict with those previously reported in the literature.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain, about 1 week postweaning, were assigned

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to seven groups of six rats each; one group was killed for carcass analysis the day the remaining rats were first fed the experimental diets outlined in table 1. Food and deionized water were fed ad libitum, and food intakes were recorded for the entire experimental period. After being fed the diets for 5 weeks, the rats were killed by enclosure in an atmosphere of carbon dioxide followed by rapid withdrawal of blood from the heart. The livers were removed and frozen immediately in liquid nitrogen. Plasma was separated from heparinized blood and stored at − 20°. The remaining carcasses were frozen after the intestinal contents had been flushed out with deionized water.

Liver and carcass nitrogen were estimated by the Kjeldahl procedure; plasma proteins by the colorimetric method of Lowry et al. (9). Total carcass fats were determined by the AOAC acid hydrolysis method (10). All magnesium estimations were done by atomic absorption spectrophotometry.[3] Readings were taken on samples of carcasses and livers which had been treated by dry ashing at 550° and suitably diluted in 0.5% HCl containing 0.1% lanthanum; plasma was diluted for analysis without previous ashing.

Liver samples were homogenized in a Potter-Elvehjem all-glass homogenizer in deionized water at 4° for enzyme analysis, and ribonuclease was measured immediately by a modification of the method of Zimmerman and Sandeen (11), using yeast ribonucleic acid as the substrate at pH 7.6. The same procedure was used for the estimation of the plasma enzyme.

RESULTS

Food intake, growth and magnesium deficiency. At all levels of magnesium intake, growth rates were uniformly greater in animals fed the 36% casein diets (fig. 1). Magnesium intake also affected weight gain at each level of dietary protein. Although, of the groups fed the 12%-casein diets, only group A (0.01% Mg) has a significantly lower final average body weight than the groups on the two higher magnesium intakes; rats fed the 36%-casein diet had progressively accelerated growth rates with each increased level of dietary magnesium.

The incidence of deficiency signs and weight gains in relation to food intake are summarized in table 2. Four rats in group A (12% casein and 0.01% magnesium) showed early vasodilation of the ears and extremities, followed eventually by the appearance of denuded areas which were most marked around the eyes and ears. All the rats in group D (36% casein and 0.01% magnesium) developed signs of deficiency, four of these being markedly more affected than the rats in group A. Weight gains in general reflected food intakes which were lower for all rats fed 12%-casein than for those fed the 36%-casein diets. When expressed in grams/100 g food intake, the weight gains in

[3] Model 290, Perkin-Elmer Corporation, Norwalk, Conn.

TABLE 1

Experimental diets

	Diet [1]					
	A	B	C	D	E	F
	%	%	%	%	%	%
Casein [2]	12	12	12	36	36	36
Mg [3]	0.01	0.05	0.10	0.01	0.05	0.10
Dextrose	71	71	71	47	47	47

[1] All diets contained methyl cellulose (2%), corn oil (8%), mineral mix (4%) and vitamin mix (2%). Jones Foster mineral mix (magnesium free) supplied per 100 g diet (in milligrams) NaCl, 558; KH_2PO_4, 1552; $CaCO_3$, 1527; $FeSO_4 \cdot 7H_2O$, 108; KI, 3.7; $MnSO_4 \cdot 2H_2O$, 17.8; $ZnCl_2$, 1.04; $CuSO_4 \cdot 5H_2O$, 1.9; and $CoCl_2 \cdot 6H_2O$, 0.91. Vitamin mix (Nutritional Biochemicals Corporation, Cleveland, Ohio) supplied per 45.45 kg diet (in grams) α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline·HCl, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 0.00135; vitamin A, 900,000 IU; and vitamin D, 100,000 IU.

[2] Vitamin-free, Nutritional Biochemicals Corporation.

[3] $MgSO_4$.

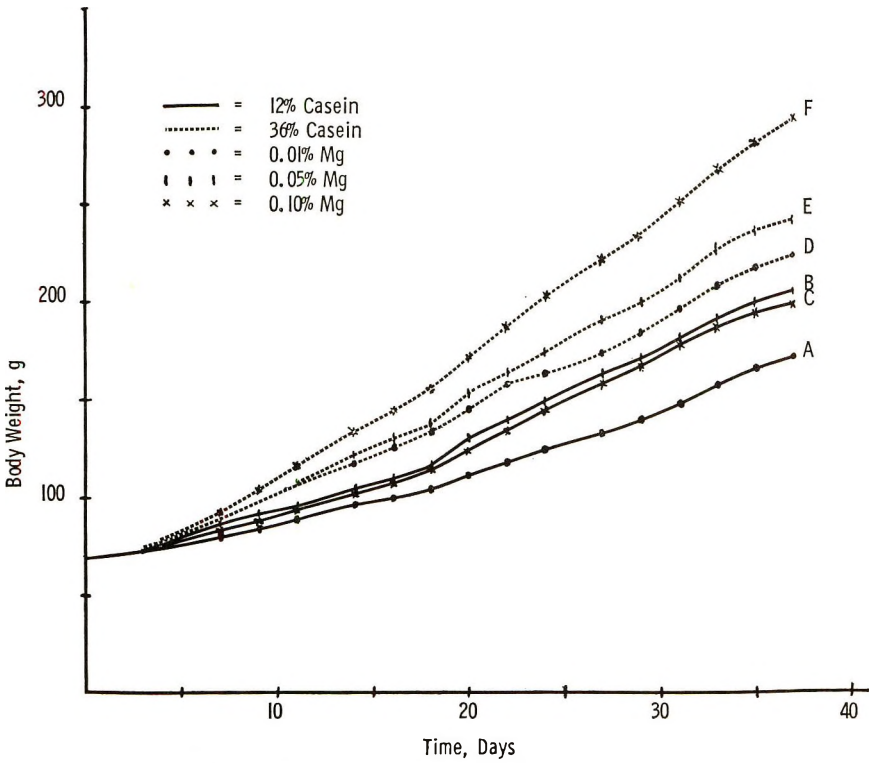

Fig. 1 Growth rates of rats on experimental diets.

groups A, B and C (12% casein with 0.01, 0.05 and 0.1% magnesium, respectively) were not significantly different. Weight gains/100 g of food eaten were significantly greater in all groups on the 36%-casein diet than in those fed 12% casein and increased with each increasing level of dietary magnesium.

Table 2 also summarizes the nitrogen and fat content of the animals at the end of the experiment. Carcass nitrogen was somewhat elevated in all groups on the 36%-casein diet, but was not significantly affected by dietary magnesium at either protein intake. Fat content was lower in all rats fed the 36%-casein diet than in the animals fed 12% casein with 0.05 or 0.1% magnesium. Among the groups fed the 12%-casein diets, the animals with the lowest intake of magnesium (group A) had a significantly lower carcass fat content. Whether this was a specific effect of magnesium intake or due merely to reduced food consumption cannot be ascertained now.

Nitrogen retention and utilization. Values for net nitrogen utilization (table 3) show essentially the same pattern of response to dietary treatment as those described for overall weight gains. The percentage retention of dietary nitrogen was not significantly different for the three groups fed the 12%-casein diets. The groups fed the 36%-casein diets showed increasing values for net nitrogen retention with each increasing level of dietary magnesium, with a very significant difference ($P < 0.01$) between groups D and F.

Liver nitrogen content appeared to be affected only by the level of dietary protein, regardless of magnesium intake, and was somewhat increased in the rats fed the 36%-casein diets. Plasma proteins were affected by both protein and magnesium intake. Plasma protein levels were not statistically different in the three groups fed the 12%-casein diets but were increased with each increasing level of magnesium in the animals fed the 36%-casein diets.

TABLE 2

Deficiency signs, food intake, weight gain, carcass nitrogen and lipid content

	Diet [1]					
	A	B	C	D	E	F
Casein in diet, %	12	12	12	36	36	36
Mg in diet, %	0.01	0.05	0.10	0.01	0.05	0.10
No. of rats per group	6	6	6	6	6	6
No. of rats showing deficiency signs	4	0	0	6	0	0
Cumulative food intake, g	468 ± 65 [2]	* 559 ± 30	* 566 ± 23	545 ± 60	557 ± 54	* 616 ± 29
Cumulative weight gain, g	103 ± 21.5	135.2 ± 9	130.2 ± 17.8	† 155.7 ± 27.0	171.3 ± 35.5	* 228.8 ± 16.6
Weight gain per 100 g food consumed	21.9 ± 1.8	24.2 ± 2.1	22.9 ± 1.9	† 28.5 ± 3.4	30.5 ± 3.3	* 36.5 ± 3.1
Carcass N, %	3.03 ± 0.11	2.92 ± 0.04	2.91 ± 0.10	3.16 ± 0.16	3.31 ± 0.15	3.20 ± 0.20
Carcass fat, %	† 13.8 ± 3.3	* 18.5 ± 1.6	* 18.5 ± 2.2	13.3 ± 5.0	12.8 ± 3.6	14.6 ± 4.3

[1] All animals were fed the experimental diets for 5 weeks.

* Significantly different ($P < 0.01$) from mean of group with the same protein intake, but with 0.01% Mg.

† Significantly different ($P < 0.01$) from mean of group C, fed 12% casein and 0.10% Mg.

TABLE 3

Net nitrogen utilization, plasma proteins and liver nitrogen concentration

	Diet					
	A	B	C	D	E	F
Casein in diet, %	12	12	12	36	36	36
Mg in diet, %	0.01	0.05	0.10	0.01	0.05	0.10
Dietary N retained, %	30.7 ±3.1 [1]	33.6 ±3.3	31.0 ±2.0	14.6 ±1.8	16.6 ±2.1	** 18.8 ±2.5
Plasma protein, g/100 ml	5.78±0.29	6.11±0.47	6.11±0.53	5.68±0.75	6.24±0.35	* 6.77±0.44
Liver N, g/100 g	2.99±0.25	2.97±0.21	3.02±0.29	3.34±0.20	3.80±0.44	3.40±0.09

[1] Mean ± SD.
* Significantly different ($P < 0.05$) from mean of group D.
** Significantly different ($P < 0.01$) from mean of group D.

Magnesium retention and concentration. The percentage retention of dietary magnesium decreased with increasing magnesium intake (table 4) but was significantly higher in animals fed 36% casein than in those fed 12%-casein diets at each level of dietary magnesium. This increased retention was evident even at the lowest level of dietary magnesium. Carcass and plasma magnesium concentrations were somewhat reduced but not significantly in rats fed the high protein intake with 0.01% dietary magnesium. At each of the other levels of magnesium intake, plasma and carcass magnesium concentrations were higher in animals fed 36% casein. Plasma magnesium was increased also with increasing magnesium intakes at both levels of dietary protein. Liver magnesium concentrations were virtually the same in all groups and appear to be minimally affected by either protein or magnesium content of the diet.

Liver and plasma ribonuclease. Liver and plasma ribonuclease levels are shown in table 5. In agreement with the reports of Allison et al. (7, 8), the levels of the enzyme in the liver and plasma were decreased in rats fed the high protein diets. An exception to this was group D (0.01% magnesium and 36% casein), in which ribonuclease levels were similar to those in groups A, B and C, fed the 12%-casein diets. In general, plasma and liver ribonuclease levels were lowered when overall nitrogen utilization was high, whether the changes in nitrogen utilization were caused by nitrogen or magnesium intake. The extent of the differences between groups, however, was different in liver and plasma. Levels in the livers of rats fed the 36%-casein diets decreased progressively with increasing magnesium intake, but differences between means were small. In the plasma the difference between groups D and E was almost 100%; there was no further difference between groups E and F.

Relationship of ribonuclease levels to parameters of protein and magnesium status. Since one of the objectives of this investigation was to test the usefulness of plasma ribonuclease as a parameter of metabolic response to dietary protein-magnesium interactions, a number of correlations of the plasma enzyme level with various other metabolic parameters were attempted (table 6). Plasma ribonuclease correlated significantly ($r = -0.72$) with plasma proteins, confirming the expected inverse relationship of ribonuclease and protein status. Unexpectedly, plasma proteins and magnesium concentration showed a significant positive correlation; consequently, plasma ribonuclease was negatively correlated with plasma magnesium. We can offer no explanation for such a relationship.

DISCUSSION

The data reported here agree with previous reports in confirming the tendency

TABLE 4

Cumulative Mg retention and carcass, plasma and liver Mg concentration [1]

	Diet					
	A	D	B	E	C	F
Casein in diet, %	12	36	12	36	12	36
Mg in diet, %	0.01	0.01	0.05	0.05	0.10	0.10
Dietary Mg retained, %	30.9 ± 5.8 [2]	39.1 ± 9.0	12.5 ± 1.6	** 18.8 ± 2.8	6.1 ± 0.6	** 12.5 ± 2.0
Mg retained, mg/100 g weight gain	14.0 ± 2.8	13.7 ± 1.9	26.1 ± 2.5	** 31.0 ± 2.7	26.8 ± 3.9	** 33.1 ± 4.4
Carcass Mg, mg/100 g	24.8 ± 2.0	22.2 ± 1.5	31.5 ± 1.2	** 35.5 ± 1.1	32.2 ± 2.3	** 37.9 ± 3.7
Plasma Mg, mg/100 g	1.35 ± 0.37	1.08 ± 0.34	1.71 ± 0.32	* 2.19 ± 0.54	2.00 ± 0.39	* 2.53 ± 0.45
Liver Mg, mg/100 g	20.4 ± 2.1	21.9 ± 0.7	22.1 ± 0.8	23.0 ± 1.5	21.8 ± 2.2	23.4 ± 0.9

[1] All comparisons are between mean values of groups on the same magnesium intake and at the two levels of dietary protein.
[2] Mean ± SD.
* Significant difference ($P < 0.05$).
** Significant difference ($P < 0.01$).

TABLE 5

Liver and plasma ribonuclease

	Diet					
	A	B	C	D	E	F
Casein in diet, %	12	12	12	36	36	36
Mg in diet, %	0.01	0.05	0.10	0.01	0.05	0.10
Liver RNase, Δ_{OD}/15 minutes per g protein	0.92 ± 0.25 [1]	1.00 ± 0.10	1.19 ± 0.27	0.99 ± 0.31	0.85 ± 0.24	0.71 ± 0.24
Plasma RNase, Δ_{OD}/15 minutes per g protein	10.84 ± 1.00	11.34 ± 2.00	11.40 ± 2.87	12.14 ± 4.77	* 6.13 ± 2.35	** 6.73 ± 1.35

[1] Mean ± SD.
* Significantly different ($P < 0.05$) from mean of group D.
** Significantly different ($P < 0.01$) from mean of group D.

TABLE 6

Correlation of some parameters of protein and magnesium status

Parameters	Correlation coefficient r	F	P
Plasma ribonuclease versus liver ribonuclease	0.57	16.0	0.005
Liver ribonuclease versus liver protein	−0.25	2.2	ns
Plasma ribonuclease versus plasma proteins	−0.72	35.9	0.005
Plasma ribonuclease versus plasma magnesium	0.66	24.5	0.005
Plasma ribonuclease versus plasma magnesium	−0.61	18.0	0.005

toward greater magnesium depletion and enhanced signs of magnesium deficiency in animals fed high protein diets with marginal levels of dietary magnesium. They also confirm the increase in magnesium requirements with increased protein intakes for maximum growth and nitrogen utilization. These data conflict, however, with those reported by Bunce et al. (4) which showed that high protein intakes increased total losses of magnesium from the body. In this investigation at all levels of dietary magnesium, the higher protein intake resulted in greater total and percentage retention of magnesium. Furthermore, effects of a low magnesium intake on the liver were negligible in this study, whereas Bunce et al. (4) reported considerable reductions in liver magnesium concentration with 0.01 and 0.02% magnesium levels.

Some of these discrepancies may be explained on the basis of differences in experimental conditions. The rats used in this investigation were of a different strain and about 25 g heavier at the beginning of the experimental period than those used by Bunce et al. (4), and the experimental period was shorter by one week. In this investigation the growth rates of rats fed the 36%-casein diet with 0.1% magnesium did not fall below those of animals fed the 12%-casein diets, nor did food intakes decrease substantially at any time throughout the experimental period. It is possible that differences in components of the diet other than magnesium and casein in the two investigations could have elicited different responses to two sets of diets that were comparable in magnesium and protein levels. Calcium and phosphorus, the two nutrients most likely to affect magnesium utilization, were fed at similar levels in both sets of investigations. This does not, however, preclude the possibility of interactions with other nutrients which have not yet been studied in relation to magnesium metabolism.

The most likely explanation for the differences in magnesium retention found in the two investigations lies in differences in methodology. The data presented in this communication are based on total carcass analyses and retentions for the entire experimental period, whereas those of Bunce et al. (4) were obtained by the balance technique for three separate 5-day periods, during weeks 2, 4 and 6 of the investigation. In addition, Bunce et al. (4), whose study was done before atomic absorption spectrophotometry became available, carried out their magnesium analyses by a method based on complexing with EDTA, a procedure considerably less specific and sensitive than atomic absorption spectrophotometry.

Since this investigation was initially planned to supplement a human metabolic study, the first objective was not to produce a condition of acute deficiency, but to demonstrate changes due to protein-magnesium interactions under milder conditions. Acute magnesium deficiency is rare in man, but there is evidence that diets, such as those customary in this country, may not supply magnesium in adequate amounts (12) though supplying other nutrients such as protein in optimal quantities for growth. Imbalances of this type, particularly during critical periods of growth, may set up abnormal metabolic patterns which may result later in life

in degenerative disease. Magnesium deficiency in rats has been shown to result in atheromatous lesions and kidney stones (13–15). Some epidemiological evidence suggests that atherosclerosis and ischemic heart disease in man may be linked with diets low in magnesium (16, 17). Such diets are also usually high in protein thus possibly contributing to a marginal magnesium deficiency, if a high protein intake were to affect magnesium retention adversely.

The pattern apparent from this study concerning the effect of a high protein intake on magnesium utilization shows that a high protein diet, by stimulating food intake and growth, may result in accelerated magnesium depletion of the total body, when magnesium is fed in limiting amounts. This depletion appears to be offset by a tendency toward greater total magnesium retention with a high protein intake, at all levels of magnesium investigated, at least while food intake is adequate. The livers, under all the conditions tested, showed no sign of magnesium depletion, whereas the total carcass content varied markedly with magnesium intake; this fact tempts one to speculate that magnesium may be preferentially preserved in tissues and cellular sites of active protein synthesis, perhaps at the expense of other metabolic processes which also require magnesium. The distribution of magnesium in different tissues under these conditions is now under investigation.

In view of the minimal changes in protein and magnesium content of the liver, a significant correlation of magnesium intake and serum proteins was unexpected. A reduction of plasma proteins usually indicates a disturbance of one of the functions of the liver, the synthesis of plasma albumin. The biosynthesis of albumin under these nutritional conditions is also under investigation in this laboratory. Preliminary data show it to be depressed at a high dietary protein level with limiting intakes of magnesium. Since magnesium is a cofactor for many vital reactions, further study of the relative effects of a protein-magnesium imbalance on metabolic key reactions should aid in the understanding of the effects of a possible marginal deficiency in human nutrition with long-term health implications.

ACKNOWLEDGMENT

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Dimers and Trimers of α-Tocopherol: Metabolic and synthetic studies

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ABSTRACT The metabolism of α-tocopherol was studied in rats and mice. Evidence has been presented for formation of dimers and trimers of α-tocopherol in animals that received parenteral α-tocopherol. These products were shown to be identical with synthetic products of oxidative condensation of α-tocopherol. The structure of these synthetic compounds was extensively studied by gas-liquid chromatography and mass spectrometry providing evidence of the necessity for modification of previously proposed structures. Dimers and trimers were not demonstrable after oral administration of α-tocopherol to rats. It is suggested that oxidative condensation does not represent a quantitatively important metabolic pathway under physiological conditions.

The metabolic fate of α-tocopherol has been studied recently by several groups of investigators (1–7). Their work has led to widely differing and sometimes incompatible conclusions. Because the basic function of α-tocopherol is still not known, we thought that further elucidation of its metabolic fate might clarify its physiological function. The most widely accepted view of the role of α-tocopherol as that of a general antioxidant suggested that elucidation of metabolic pathways involving oxidation of α-tocopherol could provide evidence for such a function.

Since omega oxidation of saturated hydrocarbons has been demonstrated in liver (8), metabolic oxidation of the side chain of α-tocopherol would seem plausible. In fact, tocopheronic acid and tocopheronolactone (Simon's metabolites) were demonstrated in the urine of rabbits and humans after very large doses of α-tocopherol (1, 2). After doses in a lower and more physiological range, however, Krishnamurthy and Bieri (6) found only 0.5% of an administered dose excreted as Simon's acid. Thus, the pathway delineated by Simon appears not to be a major pathway under physiological conditions.

Tocopherolquinone, a product of chromanol oxidation, has been reported to be a metabolic product by a number of laboratories (7, 9). It has been stressed, however, that this oxidation product can also arise as an artifact formed during isolation procedures. Thus, it is not satisfactorily demonstrated that this is a quantitatively important metabolite although the most carefully controlled studies (7) suggest that some conversion via this pathway takes place.

Martius and Furer (3) presented evidence for the presence of 2,5,6-trimethyl-3-(farnesylfarnesylgeranyl-geranyl) 1,4-benzoquinone, a product of side-chain oxidation and subsequent elongation, in tissues of rat, chick and earthworm. These findings have not been confirmed by others.

Perhaps the most widely accepted metabolic products of α-tocopherol at present are dimers and trimers formed in oxidative condensation reactions (5, 10). Alaupovic and co-workers (4) isolated three metabolites from rat and pig liver after intraperitoneal injection of α-tocopherol. One of these, "compound O," was later purified by Csallany and Draper (5, 11) who showed it to be identical with a product prepared in vitro by alkaline ferricyanide oxidation of α-tocopherol.

Later studies by Draper and co-workers, under controlled isolation conditions, clearly showed formation of both dimers and trimers after intraperitoneal injection of vitamin E to deficient rats (10). Plack and Bieri (7), however, were unable to show significant dimer formation in intraperitoneally injected rats that had been on a vitamin E-deficient diet for 1 to 5 months.

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Oral feeding by Mellors and Barnes (12) of an unspecified amount of ^{14}C-labeled tocopherol to normal rats resulted in accumulation of close to 4% of the radioactivity in the liver after 24 hours, of which 10 to 13% had the chromatographic properties of Alaupovic's compound O. However, the material was not further characterized. Krishnamurthy and Bieri (6) were unable to find dimers after oral feeding to vitamin E-deficient rats.

The current metabolic studies were designed to study the occurrence of metabolic oxidative condensation of α-tocopherol, as well as possible occurrence of Simon's pathway in the liver. We have initially attempted to confirm and extend previous studies of synthetic dimers and trimers of α-tocopherol, which were reported to be identical with the products of biological oxidation in vivo (5, 11).

The structure of a synthetic dimer of α-tocopherol, prepared by alkaline ferricyanide oxidation, has been reported by Csallany and Draper (5, 11) to be the same as a metabolic product. Draper's own studies employing ultraviolet absorption, infrared absorption and chemical modification through reduction with $LiAlH_4$ and other reagents as well as reoxidation and bromination, were interpreted as supporting structure 1 in figure 1 (11). Similar experiments by Nelan and Robeson (13) and by Schudel et al. (14) were interpreted to support structure 2 in figure 1. Schudel's studies relied on analogy of the structure of α-tocopherol dimer to the synthetic dimer of 6-hydroxy-2,2,5,7,8-pentamethylchroman, a compound differing from α-tocopherol by the absence of the phytyl side chain. Data on these compounds show the oxidative condensation products to be more complex than previously suspected.

MATERIALS AND METHODS

D,L-α-Tocopherol-5-methyl-^{14}C-acetate (specific activity, 13.1 μCi/mg) was received as a gift.[1] Free ^{14}C-tocopherol was prepared by $LiAlH_4$ reduction of the acetate in ether followed by purification on 1000-μ thick silica-gel plates with rhodamine 6-G incorporated for detection of lipids. The compound 6-hydroxy,2,2,5,7,8-pentamethylchroman was also a gift.[2] Unlabeled tocopherol was obtained commercially.[3] Normal male rats and mice were used; the animals were fed a commercial laboratory ration.[4]

For intravenous injection, 1 μCi (60 μg) of ^{14}C-tocopherol with 1 mg carrier added tocopherol were emulsified in 2:1:8, ediol–ethanol–normal saline. In oral feeding, 1 μCi (60 μg) of ^{14}C-tocopherol was emulsified in Tween 20 and normal saline without carrier.

Infrared absorption spectrum was recorded on a spectrophotometer[5] in $CHCl_3$ solvent. Nuclear magnetic resonance spectrum was recorded[6] in $CdCl_3$ relative to tetramethylsilane ($\delta = 0.00$) at 100 megacycles. Ultraviolet absorption spectrum was recorded on a recording spectrophotometer.[7]

Isolation and separation of metabolites. Animals were killed at specified times and their livers were removed; the livers were minced and extracted with 20 × volume of 2:1 chloroform–methanol for 12 hours followed by phase separation with 0.20 the volume of water. Aliquots of the $CHCl_3$ layer were taken for counting in a liquid scintillation spectrometer.[8] The remaining solutions were then evaporated to appropriate volumes over steam under a stream of nitrogen. In some experiments these were then chromatographed on silica-gel plates. The plates were developed in 20:100:1, ether–hexane–glacial acetic acid. The radioactive chromatographic fraction less polar than triglycerides and α-tocopherol was eluted with benzene from the silica gel and an aliquot counted. Further studies (see later section) showed that this nonpolar radioactivity, when present, represented dimers and trimers.

In all experiments, when tocopherol-treated animals were killed, a control animal was also killed. Its liver was removed, minced and extracted with 20 × volume of 2:1, chloroform–methanol. Ten percent of the dose of ^{14}C-tocopherol administered

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[1] From F. Hoffmann-LaRoche and Company, Ltd.
[2] From Dr. Louis Cohen, National Institutes of Health, Bethesda, Maryland.
[3] Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.
[4] Purina Rat Chow, Ralston Purina Company, St. Louis, Mo.
[5] Perkin-Elmer 21, Perkin-Elmer Corporation, Norwalk, Conn.
[6] Varian HR, Varian, Palo Alto, Calif.
[7] Cary Instruments, Monrovia, Calif.
[8] Packard Instrument Company, Inc., Downers Grove, Ill.

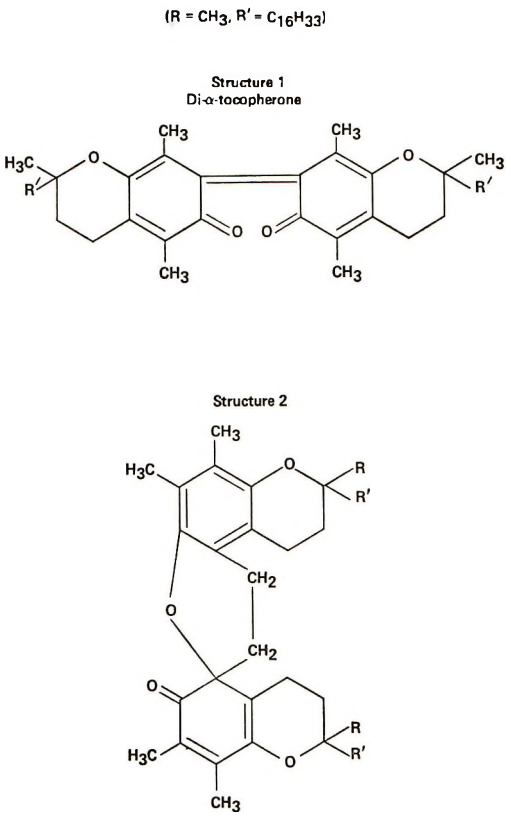


Fig. 1 Proposed structures of α-tocopherol dimer.

to each of the test animals was added to this chloroform–methanol extract at the start of the extraction procedure. The control was then handled in the same way as the test material.

The isolated liver lipids in some experiments were also treated by methods designed to demonstrate the occurrence of tocopheronic acid. Saponifiable lipids were extracted with strong base in the presence of pyrogallol; this fraction was then reacidified and extracted into hexane. In other experiments, the liver lipids were extracted with weak alkaline ethanol, according to the method of Borgstrom (15).

Results of metabolic studies. Table 1 shows that in the mouse, 2.7 to 22.9% of the intravenously injected material was recovered in the liver at the time intervals shown. The percentage of liver radioactivity present in the fraction less polar than α-tocopherol and triglycerides rises from 15% at 48 hours to 39% at 168 hours. There was no significant radioactivity in this fraction in the controls.

Table 2 shows similar results in a series of rats killed at various time intervals. Again, controls had no significant radioactivity in the fraction less polar than α-tocopherol and triglycerides.

Table 3 indicates results of feeding tracer amounts of radioactive tocopherol by oral-gastric tube. Neither experiment re-

TABLE 1

Formation of dimers and trimers in mouse liver after intravenous injection of α-tocopherol [1]

Exp. no.	Hours before killing	Injected radioactivity recovered in liver	Liver radioactivity recovered as dimer and trimer
		%	%
1	48	8.7	15
2	60	11.7	18.8
3	108	22.9	27.4
4	108	16.0	33
5	120	4.5	31
6	168	2.7	39

[1] In each experiment one mouse was injected intravenously with ^{14}C-α-tocopherol. See text for experimental details.

TABLE 2

Formation of dimers and trimers in rat liver after intravenous injection [1]

Exp. no.	Hours before killing	Injected radioactivity recovered in liver	Liver radioactivity recovered as dimer and trimer
		%	%
1	24	5.6	10
2	48	15	16.7
3	60	8.7	38

[1] In each experiment one rat was injected intravenously with ^{14}C-α-tocopherol. See text for experimental details.

TABLE 3

Formation of dimers and trimers in rat liver after feeding [1]

Exp. no.	Hours before killing	Fed radioactivity recovered in liver	Liver radioactivity recovered as dimer and trimer
		%	%
1	48	1.0	0.3
2	48	1.1	0.2

[1] In each experiment one rat was fed ^{14}C-α-tocopherol by tube. After 48 hours the rats were killed. See text for experimental details.

sulted in significant radioactivity amounting to more than 1% of the total recovered in the nonpolar fraction.

Because rapid turnover of the radioactive component of the nonpolar fraction (later shown to consist mainly of dimer) could account for the absence of the component in the feeding experiments, the metabolism of radioactive synthetic dimer (preparation described in a later section) was studied. One milligram (0.05 μCi) of radioactive synthetic dimer was injected intravenously into two mice. After 48 hours, 10.3% of the dose was recovered in pooled liver lipid extract in a fraction having the same chromatographic properties as the injected dimer.

The absence of any measurable endogenous pool of dimer in liver was shown by an experiment in which the lipids of 100 g of dog liver were extracted and the nonpolar fraction was isolated by a chromatographic procedure. No dimer or trimer was detected in this extract and, considering the sensitivity of the detection method, the amount possibly present could not exceed 0.03 μg/g liver tissue.

Studies of synthetic dimers and trimers. Alpha-tocopherol was oxidized in alkaline ferricyanide according to the method of Csallany and Draper (5). The solvent extract of the reaction mixture was purified on silica-gel plates using 5% ether-hexane as developing solvent. The various bands detected by rhodamine dye were eluted with benzene for further study. A number of bands of material less polar than α-tocopherol was formed. Table 4 shows the R_F and yields in a representative experiment in which ^{14}C-tocopherol was oxidized; the various thin-layer chromatographic bands were eluted and aliquots counted.

TABLE 4

Products of alkaline ferricyanide oxidation of ^{14}C-α-tocopherol [1]

Band	R_F	Starting material
		%
S-1	0.388	6.0
S-2	0.288	12.9
S-3	0.214	47.0
α-Tocopherol	0.125	

[1] Two million counts of ^{14}C-α-tocopherol (30 mg) were oxidized with alkaline ferricyanide. The crude product was purified on a silica-gel thin-layer plate using 5% ether-hexane solvent. The fractions less polar than α-tocopherol were eluted and aliquots were counted.

The major fraction (S-3) of the oxidation product was a yellow oil. The ultraviolet absorption spectrum revealed an absorption maximum at 296 mμ ($E^{1\%}_{1\,cm}$ = 41.8). The infrared absorption spectrum (fig. 2) revealed the absence of a hydroxyl band between 2.5 and 3.0 μ (11, 16).

The major product (S-3) could not be resolved into further components by careful thin-layer chromatography. However, S-2, on such chromatography, did resolve into two major bands (S-2A and S-2B). Also, the least polar fraction, S-1, resolved into two compounds on thin-layer chromatography (S-1A and S-1B).

Limited study of the least polar purified S-1 by mass spectrometry [9] revealed a molecular weight of 1284 (trimer-6H) which is quite compatible with a structure of a trimer proposed by Draper et al. (10). The mass spectra of S-2A, S-2B and S-3 were identical with a parent peak at m/e 858 (dimer-2H). Minor peaks were observed at 856 and 854. Decreasing the electron voltage to minimize ionization energy did not weaken these peaks relative to 858, and it is concluded that these are also parent ions.

These data appear to indicate that at least some (if not the major part) of the main dimer fraction, S-3, has a molecular weight of 858 (dimer-2H), in contrast to 856 (dimer-4H) for Schudel's proposed structure, and 828 (dimer-32H) for Draper's proposed structure (see fig. 1). Since two α-tocopherol molecules could lose only two hydrogen atoms in their combination to form a dimer with a molecular weight of 858, the possibility exists that a free hydroxyl group is present. Our infrared and nuclear magnetic resonance spectra (like those of Schudel) failed to confirm such a free hydroxyl group (fig. 3). This paradox was investigated by silylation procedures.

Bis(trimethylsilyl)acetamide (BSA) was added to the S-3 fraction at room temperature. Mass spectrometry showed a new molecular ion at m/e 930 and a prominent new ion of m/e 857. The 858 ion was ab-

[9] LKB 9000, LKB Instruments, Inc., Rockville, Md.

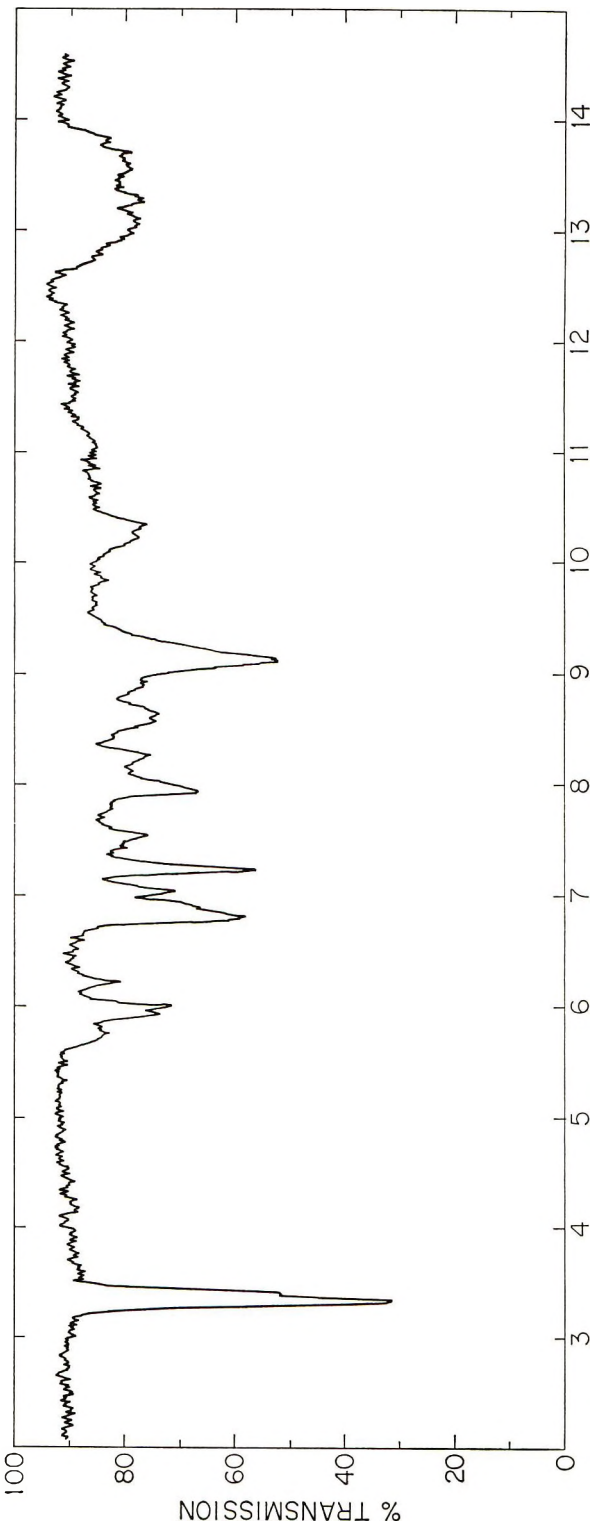


Fig. 2 Infrared spectrum of S-3 dimer fraction.

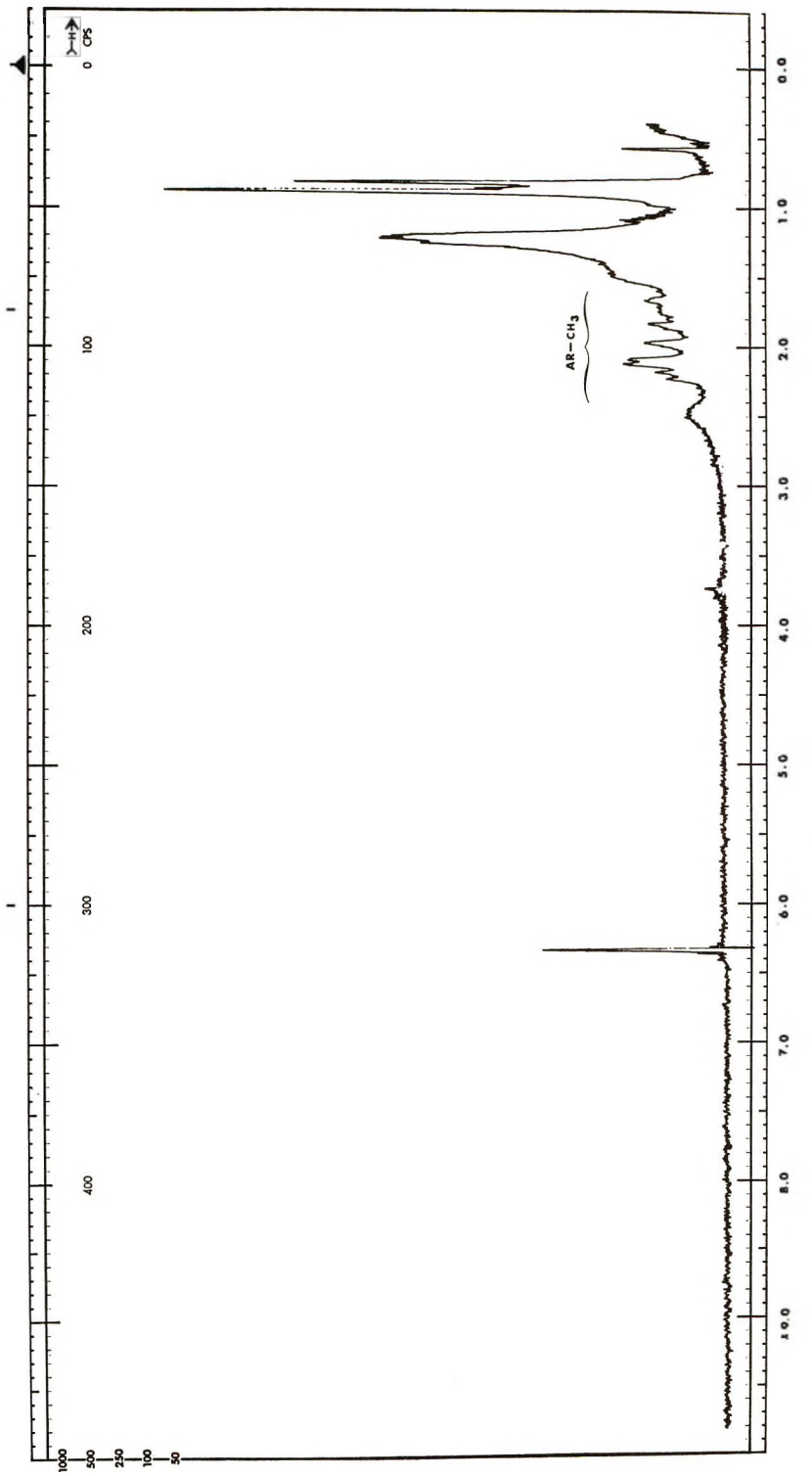


Fig. 3 Nuclear magnetic resonance spectrum of S-3 dimer fraction.

sent. These observations are consistent with the addition of one trimethylsilyl group and, consequently, the presence of one free hydroxyl group. If there is such a free hydroxyl group, one would expect that its hydrogen atom would be freely exchangeable (17). Therefore, D_2O was added to the source of the mass spectrometer, which had been allowed to equilibrate with D_2O while observing the S-3 fraction. Unexpectedly, this maneuver increased the mass of the parent peak from 858 to 860, indicating two readily exchangeable hydrogen atoms.

Since previous work had indicated that after chemical reduction the dimer has two free hydroxyls (11), we attempted to confirm this reaction. The S-3 fraction was reacted with saturated $LiAlH_4$ in ether at 0° and the products were separated by thin-layer chromatography. The two major products, both more polar than the S-3 fraction itself, had mass spectra identical with that of S-3. After reaction with bis(trimethylsilyl)acetamide, however, a new parent peak was observed at 1002 for the product of reduction of the less polar fraction and 1076 for that of the more polar fraction. This is compatible with formation of two new dimers: one with two free hydroxyls (the less polar of the two), and one with three free hydroxyls.

At this point, we began to appreciate the complexity of the situation and turned to a study of the relatively simpler model compound of α-tocopherol which lacked the phytyl side chain. The model was oxidized according to the method of Schudel et al. (14) and, after preliminary purification on a silicic acid-celite column with 1% ether–hexane as eluant, a crude product was obtained with a melting point of 88 to 91° (identical to that reported by Schudel for his precrystallization product). Preliminary attempts at crystallization were unsuccessful.

Combination gas-liquid chromatography and mass spectrometry of this product (melting point 88 to 91°) showed three major gas-liquid chromatographic peaks with parent ions at m/e 438, 434 and 434, the relative yields of which varied from experiment to experiment. It was of interest that none of these three principal components fit the structure proposed by Schudel and Isler (molecular weight 436).[10] Thus, our preparation of the model dimer confirmed the complexity of the oxidation reaction of α-tocopherol itself. The model dimer and the α-tocopherol dimer are, however, analogous in their complexity. In fact, the nuclear magnetic resonance spectrum of the S-3 fraction (fig. 3) is remarkable in the "complexity" of the aromatic methyl peaks, an observation consistent with a multicomponent nature of the α-tocopherol dimer.

The new data presented here on the structure of the α-tocopherol dimer are clearly at variance with the structure proposed by Schudel. It appears that there is a complex mixture of various α-tocopherol dimers and further work is in progress to fully resolve this problem.

Comparison of in vivo metabolites with chemically synthesized polymers. The pooled radioactive thin-layer chromatographic fraction less polar than α-tocopherol and triglycerides (see *Isolation and separation of metabolites*) was purified by two consecutive thin-layer chromatographic runs on 250-μ thick plates, first using 5% ether in hexane, and then 1% ether in hexane as developing solvents. On the last plate, three bands of material (A, B and C) were observed that contained radioactivity. Results of a representative experiment are shown in table 5.

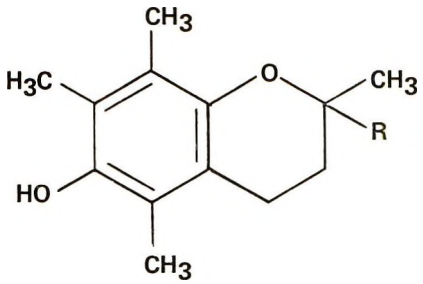
TABLE 5
Final chromatography of in vivo nonpolar metabolic products [1]

Band	R_F	Radioactivity
		%
A	0.348	24.6
B	0.600	64.0
C	0.825	11.5

[1] The partially purified radioactive fraction less polar than α-tocopherol isolated from the liver of a rat injected intravenously with labeled tocopherol was further purified by consecutive thin-layer chromatographic runs with 1% and then 10% ether-hexane as solvent. Three bands had radioactivity and the percentage of starting radioactivity in each is recorded.

The mass spectrum of band B (64% of the recovered "nonpolar" radioactivity) was identical with that of synthetic S-2

[10] A sample of crystalline model dimer was very kindly furnished by Dr. Isler of Hoffmann-LaRoche and Company. Combination gas-liquid chromatography and mass spectrometry revealed two major peaks, with apparent molecular weights of 438 and 434.



α-tocopherol
(R = $C_{16}H_{33}$)

→ Side chain oxidation-α-tocopheronic acid and α-tocopheronolactone (1,2)

→ Side chain oxidation and sequential elongation (3)

→ Chromanol oxidation-α-tocopherolquinone (7,9)

→ Oxidative condensation—dimer and trimer (5,10)

Fig. 4 Proposed pathways of α-tocopherol metabolism.

and S-3 dimer. Band B was then chromatographed after adding purified synthetic S-2 dimer. All of the recovered radioactivity was isolated with the S-2 band. Similar experiments established cochromatography of band A with S-1 trimer fraction and of band C with S-3 dimer fraction, presenting further evidence for the formation of dimer and trimer in vivo.

DISCUSSION

Various proposed metabolic processes affecting α-tocopherol are illustrated in figure 4. The results of most of the previously published studies are difficult to interpret because of use of massive doses of tocopherol (often administered parenterally) and possible modifications in structure during isolation of labile substances.

In accord with the results of others, our results provide no evidence for formation of tocopheronic acid in liver (side-chain metabolism). That such products appear in the urine seems well established, but it is difficult to assign any importance to this pathway when massive dosages are necessary for a significant demonstration.

Oxidative condensation of α-tocopherol to dimers and trimers has been studied in a number of laboratories, and although some of the authors accept the existence of such a pathway (12), others can find no evidence for it (6, 7).

Although we have demonstrated under carefully controlled conditions that oxidative condensation to dimers and trimers occurs when relatively large amounts of tocopherol are given intravenously, further evidence was necessary to establish that oxidative condensation represents a quantitatively significant metabolic pathway. Tracer amounts of α-tocopherol were fed to rats and the same methods of isolation were used as in the experiments in which the material was administered intravenously. We were unable to find any dimers or trimers. In fact, no dimer or trimer was demonstrable in 100 g of dog liver. Furthermore, metabolic studies of radioactive synthetic dimer demonstrated that the turnover of dimer is not rapid enough to account for absence of dimer in the above studies. Therefore, it is concluded that appreciable oxidative condensation occurs in vivo only when nonphysiological amounts of α-tocopherol are administered by nonphysiological routes.

ACKNOWLEDGMENTS

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Transport of Cholesterol by Blood Leukocytes and Plasma in Rabbits [1,2]

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ABSTRACT In an attempt to evaluate the role of blood leukocytes in the transport of lipids in circulation, cholesterol content and ingested exogenous cholesterol-4-^{14}C were measured in leukocytes and plasma. Cellular lipids extracted from washed leukocytes of rabbits were fractionated chromatographically and measured spectrophotometrically. The leukocytes contained 1.8 and 0.6 mg/10^9 cells of free and esterified cholesterol, respectively. Predominance of free cholesterol in leukocytes (free-ester ratio = 3:1) contrasted with predominance of esterified cholesterol in plasma (1:2.5). Twenty-two rabbits received intragastric administration of cholesterol-4-^{14}C and were killed after 4 hours, 18 hours, 7 days and 14 days. The cholesterol fractions of leukocytes and plasma were assayed by liquid scintillation counting. In leukocytes, the free fraction of labeled cholesterol was highest after 7 days, but the esterified fraction remained low throughout the 14-day period. In plasma, the labeled cholesterol was highest also after 7 days, but the esterified fraction was higher than the free throughout the experiment. The results indicate that blood leukocytes play a role in the transport of cholesterol in the circulation.

The role of blood leukocytes in the transport of lipids in the circulation has not been extensively studied. Light- and electron-microscopic studies indicate that circulating blood leukocytes carry lipid particles in their cytoplasm when animals are rendered hyperlipemic (1, 2). The purpose of the present study was to evaluate the role of circulating blood leukocytes in the transport of ingested exogenous cholesterol. Two different experiments were carried out in normal rabbits. First, the cholesterol contents of blood leukocytes and plasma were measured chemically. Second, an intragastrically administered cholesterol-4-^{14}C was extracted from blood leukocytes and plasma, isolated by thin-layer chromatography, and assayed by liquid scintillation counting.

MATERIAL AND METHODS

Measurement of leukocytic and plasma cholesterol. Arterial blood specimens were obtained from 10 New Zealand male rabbits by cardiac puncture into EDTA-containing vacuum tubes under sodium pentothal anesthesia. Blood leukocytes were suspended in 3%-dextran saline, separated from the red cell mass, exposed briefly in a hypotonic medium to hemolyze admixed red cells, and washed four times with isotonic saline. Approximately 40 IU streptokinase were added to each 1000 leukocytes of the suspension to prevent aggregation of the cells. Cell counts were made on the washed concentrated cellular suspension, using a commercial white cell dilution fluid and a hemocytometer; then leukocytic lipids were extracted with 5 volumes of chloroform–methanol (2:1). The extraction was repeated two times. All extracts were combined, filtered through benzene-cleaned filter paper, and dried under nitrogen gas. The dry residue was reconstituted in chloroform and applied on solvent-washed, heat-activated, silica-gel thin-layer plates. The thin-layer chromatograms of the unknowns and standard mixture of cholesterol and cholesteryl stearate [3] were developed using a solvent system of chloroform–benzene (3:2). The fractions of free and esterified cholesterol were scraped off separately; stained spots on the lateral sides of the plate were used as guides. The fractions were measured spec-

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[2] Presented in part at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology at Atlantic City, New Jersey, in April, 1968.

[3] Sigma Chemical Company, St. Louis, Mo.

trophotometrically after oxidation with potassium dichromate (3).

Plasma cholesterol was extracted with acetone–ethanol (1:1). Free cholesterol was precipitated with 1% digitonin solution and washed with acetone. Dry residues of the total and free cholesterol were dissolved in acetic acid and measured spectrophotometrically after reacting with ferric chloride-sulfuric acid solution (4). The concentration of esterified cholesterol was calculated by subtracting free from total cholesterol.

Measurement of cholesterol-4-^{14}C in leukocytes and plasma. Each of 22 New Zealand male rabbits weighing approximately 2 kg received an intragastric administration of 40 μCi cholesterol-4-^{14}C (specific activity 55.8 mCi/mmole) [4] dissolved in corn oil. Four, six, six and six rabbits were killed by exsanguination under sodium pentothal anesthesia at 4 hours, 18 hours, 7 days and 14 days, respectively. Lipids of blood leukocytes and plasma were extracted, purified and fractionated by thin-layer chromatography as described above. The fractions of free and esterified cholesterol of the unknowns and the cholesterol-4-^{14}C standard were scraped off the plate, eluted from silica-gel scrapings twice with chloroform-methanol and once with chloroform, gently evaporated to dryness, redissolved in toluene counting solution containing 2, 5-diphenyloxazole (PPO) and 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] - benzene (dimethyl POPOP), and counted by a liquid scintillation counter using ^{133}Ba-external standard for correction of the counting efficiency.

RESULTS

The cholesterol contents of leukocytes and plasma are summarized in table 1. The cholesterol portion of the esterified cholesterol of the leukocytes was measured using cholesteryl stearate as the standard. The results were converted to free cholesterol by multiplying by the gravimetric factor of 0.59. The quantity of leukocytic cholesterol is expressed in milligrams per 10^9 cells. This manner of expressing the quantity of leukocytic cholesterol makes a convenient comparison with plasma cholesterol levels which are commonly expressed in milligrams per 100 ml, because if a blood specimen contains 10,000 leukocytes/mm^3, which is actually close to the average leukocyte count of normal rabbit blood, then 100 ml of this whole blood would contain 10^9 cells. The free fraction of leukocytic cholesterol averaged three times the esterified fraction, whereas the levels of plasma cholesterol revealed the usual free-ester ratio of between 1:2 and 1:3.

TABLE 1

Cholesterol contents of leukocytes and plasma in rabbits [1]

	No. of rabbits	Free	Ester
Leukocytes, mg/10^9 cells	6	1.8 ± 0.7 [2]	0.6 ± 0.3 [3]
Plasma, mg/100 ml	6	15.3 ± 5.2	38.5 ± 11.9 [4]

[1] Mean body weight of rabbits is approximately 2 kg.
[2] Mean ± 1 SD.
[3] Mean is significantly lower than the value for free cholesterol ($P < 0.005$).
[4] Mean is significantly higher than the value for free cholesterol ($P < 0.005$).

The results of the radioassay on free and esterified cholesterol of the leukocytes and plasma during the 14-day period are expressed in percentage of the administered dose per 10^9 cells and per 100 ml, respectively (fig. 1). In leukocytes, the average value of the labeled cholesterol in free form was consistently higher than that in ester form. The concentration of the labeled free cholesterol in the leukocytes was highest after 7 days and had declined by day 14 after intragastric administration, whereas the esterified fraction remained low throughout the experiment. The results of the radioassay on leukocytic cholesterol after 7 days showed a significant difference between the free and esterified fractions ($P < 0.02$). In plasma, the average value of the labeled cholesterol in ester was higher than that in free form throughout the experiment; the difference between free and ester forms was greatest at 7 days, but was not significant statistically ($P > 0.2$). Both free and esterified fractions of the labeled plasma cholesterol increased until 7 days and fell after 7 days of the experiment.

[4] New England Nuclear Corporation, Boston, Mass.

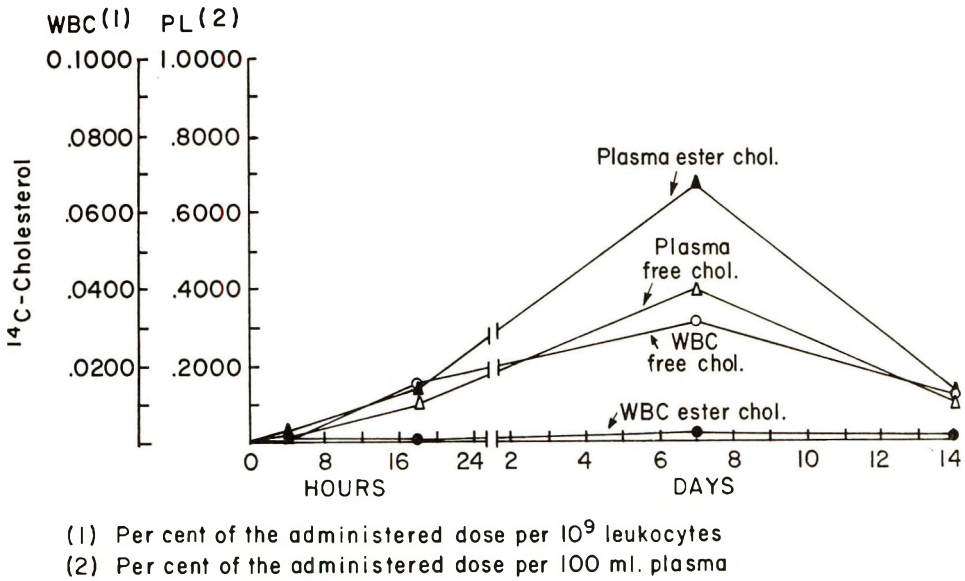

Fig. 1 The free and esterified fractions of intragastrically administered cholesterol-4-^{14}C (chol.) in blood leukocytes (WBC) and plasma (PL) after 4 hours, 18 hours, 7 days and 14 days.

DISCUSSION

The total cholesterol of rabbit leukocytes averaged 2.4 mg/10^9 cells. Whole blood leukocytes were counted in the rabbits and averaged 9706 cells/mm^3. Therefore, the total leukocytic cholesterol content was 2.3 mg/100 ml of whole blood. The total cholesterol in plasma of the same animals averaged 53.8 mg/100 ml. With an average hematocrit of 41.5% in rabbits, the total plasma cholesterol content was 31.5 mg/100 ml whole blood, making a leukocyte-plasma total cholesterol ratio of 1:14. Since the volume of white cell mass (buffy coat) comprises 1 to 2% of whole blood, the cholesterol content per unit volume of the leukocytes exceeds that of the plasma; but the content of free cholesterol is greater than that of esterified cholesterol in leukocytes, in contrast with the ester predominance in plasma.

In the present study, the washed leukocytes were mixed, and no attempt was made to separate each class of leukocytes before the cellular lipids were extracted. In our recent study in rabbits,[5] intracellular lipids stainable with oil red O were most commonly found in monocytes and lymphocytes when the animals were rendered hyperlipemic. In a previous similar study, the admixture of platelets in the washed leukocytic preparation was negligible and did not significantly influence the values of leukocytic cholesterol (5).

Rapid esterification of the absorbed exogenous cholesterol was noticed by rising levels of carbon-14-labeled esterified cholesterol in plasma. Leukocytes contained the labeled cholesterol mostly in free form despite the abundance of both free and ester forms in plasma. The labeled exogenous cholesterol in the leukocytes was highest after 7 days, but the esterified fraction consisted of only 7% of the total labeled cellular cholesterol.

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Resting Energy Metabolism in Intermittently Fed Weanling Rats [1]

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ABSTRACT In an earlier investigation, weanling rats which were alternated between 3 days of ad libitum and 3 days of partially restricted intake ingested less food while accumulating less protein and more fat than control animals fed ad libitum. Resting oxygen consumptions were measured in weanling animals fed continuously or intermittently to ascertain whether these differences were associated with a modification of the resting metabolism. Oxygen consumption of animals fed ad libitum rose from 1.00 ± 0.02 liter/hour per $kg^{0.75}$ at weaning to a value of 1.16 ± 0.03 liter/hour per $kg^{0.75}$ after 3 weeks, then gradually declined to values present initially. Resting oxygen uptakes of animals fed intermittently remained near or below 1.00 ± 0.03 liter/hour per $kg^{0.75}$ throughout this interval. Oxygen consumption of the two groups was significantly different between days 18 and 42. Suppression of a normally occurring transient rise in resting energy expenditure in the postweaning rat appears to be a part of the metabolic response of this pattern of intermittent feeding. This also suggests that normal postweaning growth of the rat is associated with a self-limiting increase in resting metabolism.

In earlier studies (1) weanling rats which were alternated between 3 days of ad libitum feeding and 3 days during which food was partially restricted differed in several physiologic characteristics from control animals fed the same diet continuously. Such animals fed intermittently gained weight at the same rate as those fed ad libitum, but with a significantly lower food intake and a higher feed efficiency. The experimental animals also accumulated less protein and more fat than did rats fed continuously. The differences in body compositions were not maintained, and carcass protein and fat of the two groups became similar as growth velocity slowed (1).

Kleiber et al. (2) observed in the rat that the period of rapid postnatal growth was associated with a transient elevation in resting metabolic rate. The resting oxygen consumption of weanling animals fed ad libitum rose progressively and at 40 days of age was 115% of that present at weaning. After this time, values declined gradually to the lower ones characteristic of the adult rat. Studies by Michels et al. (3) and Sokoloff et al. (4, 5) established that thyroid hormones stimulate protein synthesis in vivo and in vitro. A reduction in thyroid activity in weanling animals fed intermittently would account for both the reduced body nitrogen content and the enhanced food efficiency. The purpose of this investigation was to examine some of the metabolic and thyroid gland responses to continuous and intermittent feeding.

METHODS

Weanling male rats of the Holtzman strain were caged separately at 22 ± 1° with 12-hour photoperiods. Water was available at all times. The diet had the following percentage composition: dextrose, 60; fat,[3] 15; casein, 21; salt mixture (6), 4; and a complete vitamin supplement.[4] Control animals were fed ad libitum; experimental rats were alternated between 3 days with sufficient food to maintain body weight but not permit growth and 3 days of intake ad libitum. The amount of food required for weight maintenance was determined empirically and increased step-

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[2] National Institutes of Health Career Development Awardee no. 5 KO3 HD14904-07.
[3] Crisco, Procter and Gamble, Cincinnati, Ohio.
[4] Each kilogram of the diet contained vitamin A, 25,000 IU; vitamin D, 3000 IU; choline chloride, 2.25 g; and (in milligrams) α-tocopherol, 150; inositol, 150; riboflavin, 30; menadione, 68; *p*-aminobenzoic acid, 150; niacin, 130; pyridoxine·HCl, 30; thiamine·HCl, 30; Ca pantothenate, 90; biotin, 0.60; folic acid, 2.50; and vitamin B_{12}, 0.40.

wise from 4 to 10 g/day. Food intakes and body weights were recorded at 3-day intervals.

An enhanced absorptive efficiency might, in part, account for the higher food efficiency of animals fed intermittently as compared with those fed continuously. Fecal losses of fat and nitrogen were measured in control and experimental rats over a 6-day period starting after 30 days of feeding. Feces were collected from animals housed individually in stainless steel metabolism cages. Analyses of feces for fat and nitrogen content were carried out as described for carcass composition determinations.

Resting metabolic rate was measured in single animals gently restrained in a closed system containing soda lime (7). Oxygen consumption was determined from rate of volume change of the system measured at intervals over a 30- to 60-minute period. Four to six determinations were averaged to obtain values for each animal. Oxygen uptakes were corrected for temperature and pressure and recorded as milliliters per hour per kilogram body weight$^{0.75}$. Determinations were carried out at 6-day intervals in control animals. Initially, daily observations were made on experimental rats over two 6-day cycles to establish that cyclic variations related to the phase of feeding cycle were insignificant.

Resting caloric expenditures were approximated using a caloric equivalent of 4.8 kcal/liter oxygen uptake. Energy intake was estimated from diet composition using values of 4, 4 and 9 kcal/g of carbohydrate, protein and fat, respectively. Though not precise, such values were adequate for the approximations desired.

To examine further the role of the thyroid gland in the changes induced by this feeding pattern, thyroxine levels were maintained relatively constant by providing exogenous hormone to thyroidectomized animals fed continuously or intermittently. The effect of these feeding programs on thyroid weight in hemithyroidectomized animals was also examined. Thyroid-parathyroidectomy and hemithyroidectomy were carried out under ether anesthesia immediately prior to the initiation of the feeding program. Hemithyroidectomized animals were fed the standard diet. Thyroid-parathyroidectomized animals were fed this diet supplemented with 0.5 mg thyroxine/kg and were provided with 0.5%-calcium lactate solution to drink. Hemithyroidectomized rats were killed after 14 days; thyroidectomized rats were maintained for 60 days.

Upon completion of feeding studies, thyroidectomized rats were killed by an overdosage of ether; gastrointestinal fill was discarded and the carcass desiccated by lyophilization. The water-free carcass was pulverized with acetone in a blender.[5] Acetone was not discarded but removed by evaporation. Nitrogen was determined by the micro-Kjeldahl method. Aliquots of dried pulverized carcass were extracted with methanol–chloroform (1:1) in a Soxhlet extraction apparatus. Solvents were removed by evaporation, and the fraction soluble in petroleum ether–chloroform (6:1) was weighed.

RESULTS

Absorptive efficiency. Feces collected over a 6-day period starting after 30 days on the feeding program contained fat equivalent to 2.9 ± 0.3% (mean ± SE) and 2.6 ± 0.1% of that ingested during this interval by control and experimental animals, respectively. In both groups, fecal nitrogen was equivalent to 2.8 ± 0.2% of intake. Water intakes at this time were 18 ± 1 ml/day for experimental and 20 ± 2 ml/day for control animals.

Resting metabolism. Resting oxygen consumptions (milliliters per hour per kilogram$^{0.75}$) of experimental animals decreased gradually and progressively throughout the 60 days of feeding (table 1). In control animals, oxygen uptake progressively increased and reached a peak value, approximately 116% of that present at weaning, at 24 days (table 1). Oxygen uptakes of control animals then declined and on day 42 reached a value not different from that of animals of similar age fed intermittently. Resting oxygen consumptions were significantly lower in experimental as compared with control animals between days 18 and 36 (table 1).

Thyroid gland weights of experimental animals were lower than those of controls. Difference was significant only on day 24;

[5] Waring Products Company, Winsted, Conn.

TABLE 1

Body weight, thyroid weight and resting oxygen consumption in control and experimental animals [1]

Day no.	Body wt		Thyroid wt [2]		Oxygen consumption	
	Control	Experimental	Control	Experimental	Control	Experimental
	g	*g*	*mg/100 g body wt*	*mg/100 g body wt*	*liters/hr/kg*$^{0.75}$	*liters/hr/kg*$^{0.75}$
0	51 ± 1 [3]	51 ± 1	12.40 ± 1.06	12.40 ± 1.06	1.000 ± 0.020	1.000 ± 0.020
6					1.065 ± 0.020	1.062 ± 0.032
12	112 ± 4	107 ± 5	9.80 ± 0.19 [4]	10.53 ± 0.03 [4]	1.099 ± 0.024	1.030 ± 0.023
18			9.20 ± 0.20	8.91 ± 0.11	1.154 ± 0.033	0.950 ± 0.042 [5]
24	179 ± 5	173 ± 3	8.60 ± 0.27	7.85 ± 0.22 [5]	1.165 ± 0.033	0.916 ± 0.032 [5]
30			7.34 ± 0.13	7.34 ± 0.13	1.109 ± 0.030	0.976 ± 0.032 [5]
36	251 ± 3	240 ± 5	7.30 ± 0.12	6.98 ± 0.21	1.106 ± 0.024	0.913 ± 0.033 [5]
42					1.002 ± 0.030	0.917 ± 0.025
48	301 ± 9	287 ± 7	6.64 ± 0.27	6.32 ± 0.17	1.035 ± 0.030	0.982 ± 0.021
54					1.016 ± 0.028	0.913 ± 0.020
60	310 ± 8	312 ± 5	5.95 ± 0.16	5.51 ± 0.15	0.930 ± 0.015	0.914 ± 0.020

[1] All groups, unless otherwise specified, contain nine animals.
[2] Thyroid weights obtained from animals other than those used for metabolic rate studies.
[3] Mean ± SE.
[4] Six animals per group.
[5] Values for experimental group significantly lower than those for control animals ($P < 0.05$).

at that time, the resting oxygen consumption of control animals was maximal (table 1).

The total cumulative resting caloric expenditure of the two groups between days 18 and 36 differed by approximately 200 kcal. Total food intake of control animals for the 60-day period was 757 ± 11 g as compared with 680 ± 6 g for experimental animals. The 60-day intake of the group fed intermittently was approximately 350 kcal lower than that of control animals.

To assess the relationship between food intake and resting energy metabolism, total calories ingested were divided into three arbitrarily chosen intervals of 18 days starting at days 1, 21 and 42 and designated periods 1, 2 and 3, respectively. Intakes could thereby be compared during periods when the resting metabolic rates of the two groups were the same (periods 1 and 3) and different (period 2). Food intakes of control animals averaged 178 ± 4, 286 ± 12 and 234 ± 10 g in periods 1, 2 and 3, respectively. Intake was significantly higher during period 2 when resting metabolic rate was also maximally elevated. Values for experimental animals for these same intervals were 162 ± 5, 216 ± 5 and 240 ± 7 g. Intakes of experimental animals increased progressively and did not exhibit the transient elevation during period 2 that occurred in control animals. Intakes were significantly lower than those for controls during this interval when the metabolic rates also differed.

In hemithyroidectomized animals, residual thyroid tissue was significantly smaller in rats fed intermittently as compared with those fed continuously. After 14 days of feeding, remaining thyroid tissue in eight experimental animals weighed 4.9 ± 0.3 as compared with 6.6 ± 0.4 mg in eight control animals.

Metabolic rates of thyroidectomized animals fed replacement thyroxine averaged 1.08 ± 0.05 liter/hour per kg$^{0.75}$ in control rats and 1.05 ± 0.04 liter/ hour per kg$^{0.75}$ in animals fed intermittently. Nitrogen and fat content of control and experimental animals did not show the differences previously observed (1) in intact animals (table 2). When food intakes of operated animals were partitioned into the three

TABLE 2

Body weight, food consumption and carcass composition of thyroidectomized, thyroxine-supplemented animals after 60 days [1]

	Control	Experimental
	g	*g*
Body wt	258 ± 10 [2]	224 ± 11
Food intake	690 ± 16 [3]	595 ± 15
Body fat	24.7 ± 3.4	29.2 ± 2.4
Body nitrogen	8.98 ± 0.38	7.91 ± 0.35

[1] Seven animals per group.
[2] Mean ± SE.
[3] Values for control and experimental animals significantly different $P < 0.05$.

18-day periods as for intact animals, control animals ingested 148 ± 6, 223 ± 7 and 234 ± 10 g during the three periods at the beginning, middle and end of the 60 days of the experiment. Animals fed intermittently ate 110 ± 3, 202 ± 4 and 208 ± 9 g during these same intervals. Intakes of experimental rats differed from that of control rats only during period 1 and not during period 2 as in intact animals.

DISCUSSION

Fecal excretion of nitrogen and fat were unaffected by the feeding patterns employed in this study. Efficiency of assimilation did not appear to be a significant factor in the enhanced food utilization of animals fed intermittently as compared with those fed ad libitum (1). Water intakes of the two groups were also not modified by this feeding pattern.

The transient alterations in body composition observed in weanling rats fed intermittently are, to some extent, similar to those noted by other investigators (8–10) in older animals that were force-fed or allowed access to food for only a few hours each day. These compositional changes in animals that were force-fed were associated with alterations of thyroid function (11) and total physical activity (12). In contrast to the effects observed when adult rats are fed once daily, the 6-day cyclic feeding pattern employed in the present studies leads to only self-limiting alterations in body composition that are confined to the period of rapid growth.

The transient rise in resting oxygen consumption of postweaning animals fed ad libitum observed in this study showed the same time course as that observed by Kleiber et al. (2). Highest values for resting metabolism in both the present and the earlier study occurred between 40 and 45 days of age. The metabolic rates observed in this study were approximately equal to those reported by Kleiber et al. (2).

Experimental animals showed no comparable rise in resting metabolic rate, and metabolic rates declined throughout the 60 days of observation. This transient difference in resting energy expenditure appeared to partially determine food intake in the two groups. Intake of control animals was greatest when the metabolic rate was elevated. In experimental animals, no comparable changes in intake or resting metabolic rate occurred.

The differences in the degree of hyperplasia of thyroid remnant in hemithyroidectomized animals also suggest that thyroid activity was modified by intermittent feeding. The association, in rats fed intermittently, of reduced resting metabolic rate, depressed thyroid activity and decreased rate of protein accretion suggests that these are interrelated. The studies by Michels et al. (3) and Sokoloff et al. (4, 5) indicated that protein synthesis was stimulated by thyroid hormones. The transient reduction in rate of protein deposition could be causally related to the change in thyroid activity induced by intermittent feeding. Alterations in body composition did not develop in thyroidectomized animals with replacement thyroxine and fed intermittently or ad libitum; this is also suggestive that changes in thyroid activity may be related to the differing rates of protein deposition in intact animals.

Food intake of thyroidectomized animals was less than that of unoperated rats. The reduction was most pronounced immediately following surgery. Leathem (13) reported that one immediate effect of thyroidectomy is a reduction in food intake. The reduced food intake observed postoperatively in the animals fed intermittently might be due to the addition of the stress of the intermittent feeding pattern to that of surgery.

Results of this study suggest that the differences in food efficiency and body composition associated with intermittent feeding may be mediated, in part, by a modification of thyroid activity. In addition, the rate of protein accumulation in normal postweaning growth may involve transient modifications in thyroid activity.

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Studies of Diet as a Factor in ^{137}Cs Metabolism by Rats [1]

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ABSTRACT Rats fed semistarvation, high carbohydrate, high protein and high fat diets retained more ^{137}Cs than control animals 7 days after intraperitoneal injection of a single dose of the isotope. The primary effect of diet on cesium metabolism may have been modification of cesium excretion via urine and feces. There appeared to be a direct relationship between mass of fecal material and the amount of ^{137}Cs excreted in feces. Apparently diet variation changed the composition and amount of material in the intestinal lumen to which cesium could be adsorbed for subsequent excretion in feces. On the other hand, cesium excretion via urine seemed to be less dependent on the amount of urine produced. Varying the primary nutrients by feeding rats high protein, high carbohydrate or high fat diets resulted in increased or decreased affinity of some cells for cesium. These results are not readily explainable but may be due to shifts in the relative amounts of connective tissue present in the tissues studied.

This study was undertaken to observe the effect of diet on the whole-body retention and distribution of ^{137}Cs in body tissues in the laboratory rat. The diet may be expected to alter the whole body retention of cesium by changing 1) the amount and composition of excretory materials to be handled by the kidneys and gastrointestinal tract (for example, a high protein diet results in an increase in urea synthesis and excretion; a high fat diet slows the rate of movement of food through the gastrointestinal tract), and 2) the degree of ionization of intracellular proteins (for example, when glucose is replaced by amino acids or fatty acids as the major cellular metabolite, the net charges on cell proteins and affinity of protein for cesium may change).

MATERIALS AND METHODS

Male, white laboratory rats of the Cheek-Jones strain (9 to 10 weeks old) were maintained at 23 ± 2° for a minimum of 7 days before separation into experimental groups.

Experiments were divided into series 1 (diet restriction) and series 2 (diet variation). Each experimental group included 12 animals. As illustrated in figure 1, series 1 consisted of: A, a control group which was allowed an 8-day acclimation period in the metabolic cages before ^{137}Cs injection, B, a group subjected to a 5-day acclimation period followed by a 3-day starvation period before ^{137}Cs injection, and C, a group with the same preinjection schedule as group B. After isotope injection rats in group A (control) and B (preinjection starvation) were given food ad libitum, and rats in group C (semistarvation) were given 10 g food/day. The rats in series 1 were fed a commercial ration.[2] Rats in groups A, B and C were killed 7 days after isotope injection.

Rats in series 2 were fed a high carbohydrate, a high fat, a high protein or a control diet. The control diet had the same composition as the control diet for series 1. Diet compositions are presented in table 1. The animals in series 2 were allowed 10 days for acclimation to experimental diets and were permitted food and water ad libitum throughout the experiment. Following the acclimation period ^{137}Cs solution was administered to the animals of series 2 (the same dose as in series 1) and they were killed 7 days after the injection.

The ^{137}Cs (in carrier-free state) [3] was administered in a single intraperitoneal in-

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[2] Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.
[3] Obtained from Nuclear Science and Engineering Corporation, Pittsburgh, Pa.

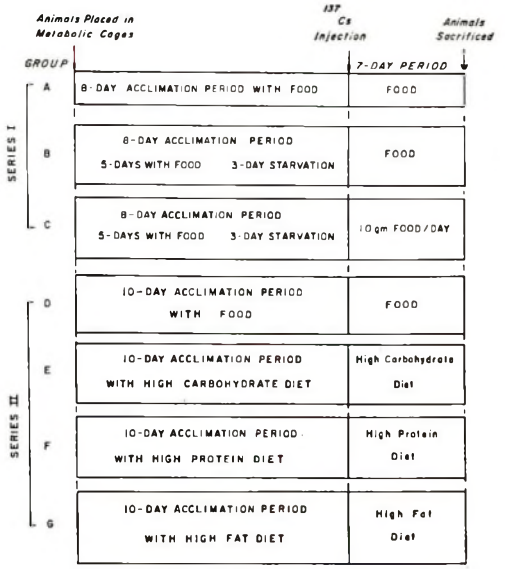

Fig. 1 Protocol of experiments.

TABLE 1

Diet compositions

Component	Wayne Lab-Blox [1]	High CHO [2]	High protein [2]	High fat [2]
	%	%	%	%
Crude protein	24	—	—	—
Protein (vitamin-free casein)	—	18	64	18
Sucrose	—	68	22	29
Vegetable oil	4	8	8	45
Brewer's yeast	—	2	2	4
Salt mixture USP XIII no 2 [3]	—	4	4	4

[1] Wayne Lab-Blox is described as a complete diet, containing all nutrients known to be needed for maintenance, reproduction and growth, containing no antibiotics or chemical preservatives (Allied Mills, Chicago, Ill.).

[2] Vitamin content of diets: (grams per kilogram) vitamin A, 0.099; vitamin D, 0.06; alpha-tocopherol, 0.11; ascorbic acid, 0.99; inositol, 0.11; choline chloride, 1.65; menadione, 0.051; *p*-aminobenzoic acid, 0.110; niacin, 0.099; riboflavin, 0.022; pyridoxine·HCl, 0.022; thiamine·HCl, 0.022; and calcium pantothenate, 0.066; and (in milligrams per kilogram) biotin, 0.43; folic acid, 1.98; and vitamin B_{12}, 0.03.

[3] Salt content of diets: (in grams per 100 g) sodium chloride, 0.23; magnesium sulfate, 0.72; sodium biphosphate, 0.46; potassium biphosphate, 1.27; calcium biphosphate, 0.72; ferric citrate, 0.16; and calcium lactate, 1.73.

jection approximately 1 cm to the left of the midventral line and halfway between the anus and ribcage. The dose was 0.15 ml/100 g body weight of a standard cesium iodide stock solution containing 1.1 μCi ^{137}Cs/ml in buffered (pH 6.8) mammalian Ringer solution. This dosage gave an initial whole-body count in the range of 170,000 to 370,000 cpm. All injections were made with a 26-gauge, 1.27-cm needle. Immediately after injection the animals were placed into cylindrical cardboard containers (8 cm diameter by 9 cm length), centered in a small animal whole-body counter,[4] and counted for 1 minute. The counting efficiency for ^{137}Cs was 25 to 30%. The time of injection, amount of injection and initial counts per minute were recorded for each animal.

To minimize error due to daily biological rhythms, weights and radioactivity measurements for the animals were all made between 6:00 and 9:00 AM. Data collected included periodic measurements of radioactivity and weights of the rats and their excreta.

Animals were decapitated; the heart, lungs, liver, spleen, stomach, small intestine sample, kidneys, right gastrocnemius, left gastrocnemius, a skin sample, both tibiae, 1 ml plasma, 1 ml blood and the brain were removed and placed in separate containers for weighing and ^{137}Cs counting. All tissues were weighed to the nearest milligram. Live animals, feces, urine and animal remains after killing were weighed to the nearest 0.5 g.

The tissue retention index (TRI) was employed to describe relative distribution of isotope in the experimental animals. Values for TRI were computed using the ratio $\frac{a}{b}$, where "a" represents the amount of isotope per gram of tissue in question and "b" represents the amount of isotope per gram of live animal at the time of killing. Values greater than 1.0 indicate a relative accumulation of isotope, whereas TRI values less than 1.0 indicate a low tissue affinity for the isotope. The TRI partially compensates for differences in tissue sizes, tissue isotope concentrations, animal size variation and the total amount of isotope in the animal; it is interpreted as a pure number indicating relative distribution of isotope in an animal. The example on next page indicates the usefulness of TRI.

[4] Packard Model 440 Armac scintillation detector equipped with a Series 410A Auto-gamma spectrometer, Packard Instrument Company, Inc., Downers Grove, Ill.

Animal	Tissue (organ)	Tissue wt, g	Tissue cpm	Tissue cpm/g	Animal cpm/g	TRI
A	Liver	10.000	10,000	1,000	500	2.0
B	Liver	15.000	15,000	1,000	1,000	1.0

The data for amount of isotope per gram of liver are the same for both animals but animal B has twice as much isotope per unit of mass as animal A. Therefore, the liver of animal A contains twice as much isotope as the liver of animal B when considering the relative distribution of isotope in the animals.

It is important to realize that changes in TRI may be due to movement of ^{137}Cs or movement of tissue components which do not attract ^{137}Cs. An organ may decrease in size by 50% while the animal decreases in size by 30%. If the weight loss from the organ is due to depletion of a substance with a low affinity for ^{137}Cs, the tissue concentration of ^{137}Cs will increase and TRI will also increase. In this example there is no shift in isotope distribution as suggested by the increase in TRI, merely an increase in concentration of the isotope due to loss of some tissue component. This information makes TRI a useful tool for predicting the causes for shifts in relative distribution of isotopes among tissue.

RESULTS

Rats for series 1 averaged 245 g (fig. 2) at the beginning of the experiment, whereas series 2 animals had an initial mean weight of approximately 190 g (fig. 3). Control animals gained weight throughout the experiment. Mean body weights of group B animals (series 1) were much less than the control group after the starvation period, but regained weight fast enough to almost equal the mean weight of control animals on day 7 after injection. The animals in group C lost weight constantly. The 10 g/day food allowance of group C animals appeared insufficient for replacement of nutrients lost during the 3-day starvation period. Animals of groups A, D, E, F and G had approximately the same average weight increase, 3 to 5 g/day, between injection and killing. Animals of

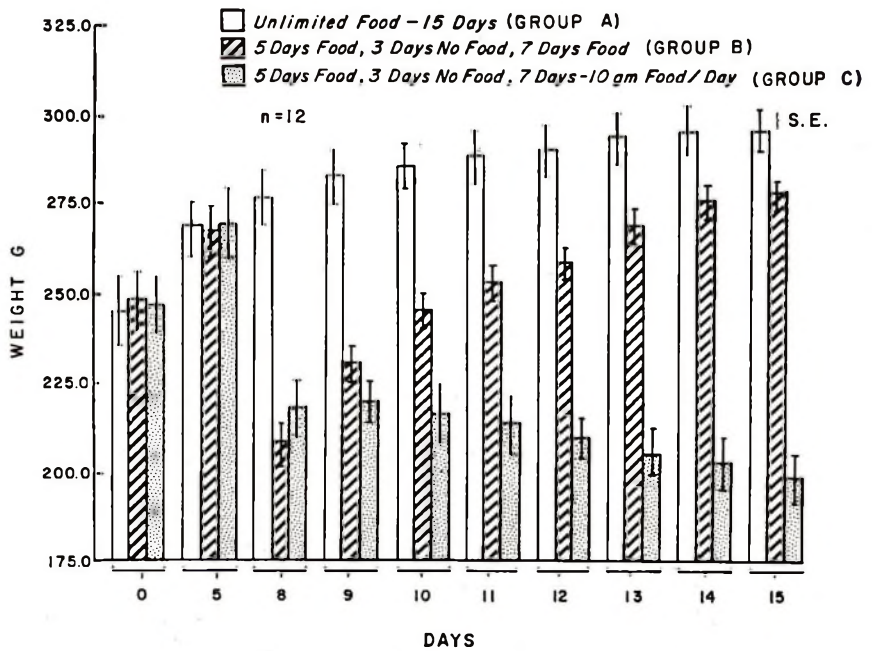

Fig. 2 Mean animal weights, series 1.

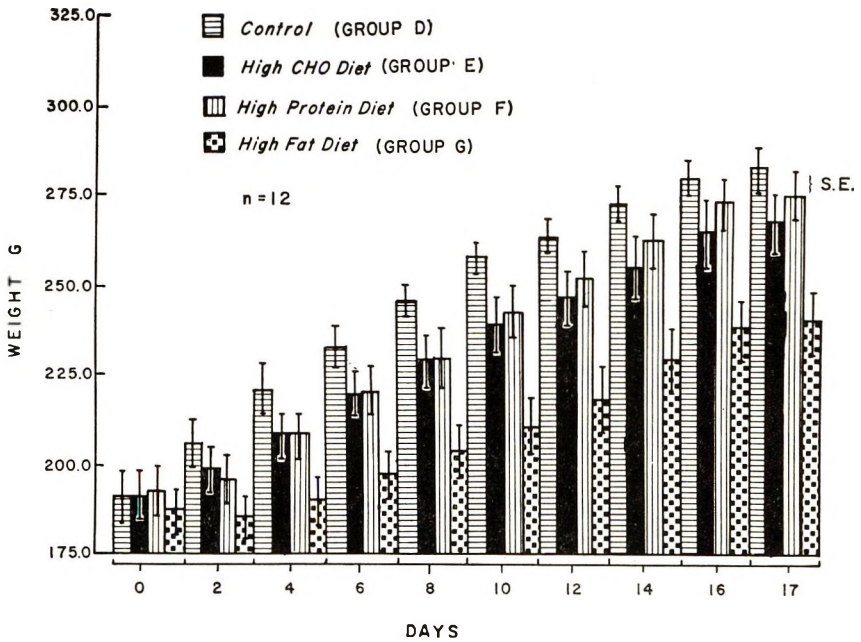

Fig. 3 Mean animal weights, series 2.

TABLE 2

Cesium retention and excretion data [1]

	Series 1			Series 2			
	A Control	B Pre-injection starved	C Semi-starved	D Control	E High CHO	F High protein	G High fat
% retention	46.7	44.6	59.6	43.1	68.5	54.2	68.2
SE	1.0	1.6	0.8	1.1	0.9	1.2	0.9
P		0.3	0.01		0.01	0.01	0.01
% in urine	41.4	44.3	33.7	44.1	29.9	45.1	29.3
SE	1.5	1.4	0.7	1.1	0.9	1.3	1.0
P		0.2	0.01		0.01	0.6	0.01
% in feces	14.2	15.5	9.8	13.3	1.3	1.8	1.4
SE	1.9	1.7	1.0	1.8	0.1	0.8	0.1
P		0.7	0.05		0.01	0.01	0.01
Total	102.2	104.4	103.2	100.4	99.7	101.0	99.0
SE	0.6	0.9	0.7	0.6	0.3	0.8	0.5
P		0.1	0.3		0.3	0.6	0.1
Urine weights, g/day postinjection							
Mean	9.5	12.5	13.0	10.5	5.0	24.0	3.0
SE	0.5	0.9	2.0	0.6	0.7	1.4	0.3
P		0.01	0.2		0.01	0.01	0.01
Feces wt, g/day postinjection							
Mean	8.5	9.5	3.5	6.5	0.5	0.5	0.5
SE	0.5	0.5	0.2	0.3	0.0	0.0	0.0
P		0.2	0.01		0.01	0.01	0.01

[1] Twelve animals in each group, *P* values for experimental and control groups within each series.

group B, however, gained 10.0 g/day and those of group C lost 2.8 g/day during the same period.

The percentage of ^{137}Cs retained (table 2) at the end of the 7 days was increased over control values by semistarvation after injection, the high carbohydrate, high protein and the high fat diets ($P < 0.01$). The counts per minute per gram values for the animals in these four groups were correspondingly high (table 3).

As indicated in table 2, animals on semistarvation, high carbohydrate and high fat diets excreted less ^{137}Cs in urine than control animals ($P < 0.01$). The amount of ^{137}Cs excreted in the feces was low for animals on the semistarvation, high carbohydrate, high protein and high fat diets (P values 0.05 to 0.01).

Regarding urine and fecal excretion per day (table 2), it is pertinent to note a) the small amount of feces excreted by rats in the high carbohydrate, high protein, high fat and semistarvation diet groups ($P < 0.01$), b) the relatively high urine excreted per day for rats in the high protein group

TABLE 3

Tissue weight and activity data [1]

Tissue	Series 1			Series 2			
	A Control	B Pre-injection starved	C Semi-starved	D Control	E High CHO	F High protein	G High fat
Tissue weight, g							
Liver	10.747	9.747	4.908	11.739	12.060	12.610	9.592
SE	0.412	0.307	0.136	0.540	0.592	0.492	0.480
P		0.1	0.01		0.8	0.3	0.01
Kidney	2.324	2.241	1.504	2.208	2.058	2.690	1.823
SE	0.058	0.048	0.040	0.046	0.080	0.087	0.045
P		0.3	0.01		0.2	0.01	0.01
Muscle	1.703	1.517	1.263	1.738	1.651	1.673	1.457
SE	0.040	0.042	0.053	0.042	0.071	0.055	0.053
P		0.01	0.01		0.4	0.4	0.01
Tissue cpm/g							
Liver	469	341	891	366	467	552	504
SE	21	14	53	15	16	22	12
P		0.01	0.01		0.01	0.01	0.01
Kidney	527	407	955	458	786	480	891
SE	20	14	34	16	22	16	32
P		0.01	0.01		0.01	0.4	0.01
Muscle	1116	977	1402	1006	1571	1238	1550
SE	44	32	29	34	19	36	35
P		0.01	0.01		0.01	0.01	0.01
TRI							
Liver	0.99	0.92	1.32	0.92	0.73	1.13	0.80
SE	0.020	0.016	0.055	0.016	0.018	0.022	0.007
P		0.02	0.01		0.01	0.01	0.01
Kidney	1.12	1.11	1.42	1.15	1.24	0.98	1.41
SE	0.016	0.021	0.038	0.012	0.021	0.017	0.032
P		0.8	0.01		0.01	0.01	0.01
Muscle	2.36	2.39	2.09	2.53	2.48	2.53	2.46
SE	0.032	0.034	0.032	0.027	0.032	0.032	0.029
P		0.6	0.01		0.3	1.0	0.1
Counts per minute per gram for live animals at killing							
Mean	472	368	672	397	635	490	632
SE	15	13	14	11	10	14	12
P		0.01	0.01		0.01	0.01	0.01

[1] Twelve animals in each group, P values for experimental and control groups within each series.

($P < 0.01$), and c) the low urine output by rats in the high carbohydrate and high fat diet groups ($P < 0.01$).

When compared with control values, the following TRI differences were observed (table 3). Animals of groups B (preinjection starved), E (high carbohydrate) and G (high fat) had low liver TRI values (P values 0.02 to 0.01), and animals of groups C (semistarved) and F (high protein) had high liver TRI values ($P < 0.01$). The animals of groups C (semistarved), E (high carbohydrate) and G (high fat) had high kidney TRI values ($P < 0.01$), whereas animals of group F (high protein) had a low kidney TRI ($P < 0.01$). Muscle TRI values for animals of group C (semistarved) were less than control values ($P < 0.01$).

DISCUSSION

Turnover rate of ^{137}Cs was related to diet as evidenced by the percent whole-body retention, urine and fecal excretion of ^{137}Cs and tissue retention index data. The differences among control and experimental groups suggest quantitative and qualitative aspects of diet affect ^{137}Cs metabolism.

Whole-body retention of ^{137}Cs was increased over control values by high carbohydrate, high protein, high fat and semistarvation diets. These differences were related to urine and fecal excretion. Animals fed high carbohydrate and high fat had reduced urine and feces output. Animals fed the high protein diet had high urine output and low feces output. Semistarved animals had urine volumes similar to control values and low feces output. Increasing urine production did not increase the amount of cesium excreted during the 7-day experimental period. Hood and Comar (1) reported 1-week ^{137}Cs excretion in the rat to be 25 to 40% in urine and 2 to 8% in feces; these data agree with data for control animals presented in table 2. The high urine production on a high protein diet confirmed the findings of Schmidt-Nielsen (2) who reported that urea clearance varied directly with the protein content of the diet. Hendrikx and Epstein (3), Osborne et al. (4), and others (5, 6) have observed that dietary protein in the rat led to hypertrophy of the kidneys and excretion of a more concentrated urine. Our rats fed the high protein diet had the expected hypertrophy of kidneys, and excretion of cesium via urine for animals fed high protein was almost exactly the same as for control animals, although urine output for animals fed high protein was approximately double that of the control animals. The high urine production with a low total excretion of ^{137}Cs has been observed during hyperthermia [5] as well as with the high protein diet. The ^{137}Cs excretion via feces appeared related to the amount of feces excreted (table 2). As suggested by Moore and Comar (7), endogenous secretion of ^{137}Cs could make the isotope available for adsorption to fecal material. Our data suggest the degree of cesium adsorption of fecal material to be dependent on the composition and quantity of adsorbing surfaces available in the intestinal lumen; this idea has been suggested by several investigators including Moore and Comar (7), Williams and Patrick (8) and Mraz and Patrick (9).

Regarding TRI values it is pertinent to note that a) liver TRI was high in animals fed the semistarvation and high protein diets, b) liver TRI was low in animals fed the preinjection starvation, high carbohydrate and high fat diets, c) kidney TRI was high in semistarved animals, animals fed the high carbohydrate diet and animals fed the high fat diet, d) kidney TRI was low for animals fed the high protein diet, and e) muscle TRI was low in semistarved animals.

The high liver TRI for animals fed the high protein diet suggests utilization of amino acids facilitates ^{137}Cs accumulation in the liver. The low liver TRI for animals fed the high carbohydrate and high fat diets suggests carbohydrate and lipid do not facilitate ^{137}Cs accumulation in the liver. Studies on isolated tissues using known concentrations of amino acids, carbohydrates and fatty acids are needed to clarify the interactions of organic and inorganic ions.

Furchner et al. (10) suggested that differences in cesium concentration in mice kidneys were due to differences in size and not to the amount of cesium in the organ;

[5] Savignac, N. 1967 Heat exposure and 137-cesium distribution in tissues of *Citellus spilosoma*. M.S. thesis, University of New Mexico, Albuquerque.

they also stated that cesium has a low affinity for fat. This suggests ^{137}Cs is bound with or attracted to hydrophilic tissue in the kidney (and possibly other organs). If the decreased kidney weight (table 3) in semistarved, high carbohydrate diet and high fat diet groups were due to mobilization of lipid, the TRI values could have been a function of kidney size; the low kidney TRI value for animals fed the high protein diet may have been the result of an increase in kidney lipid content or an increase in some kidney component other than lipid with a low affinity for ^{137}Cs. This same weight factor appeared to be responsible for the high liver TRI in the semistarved animals.

The low muscle TRI for semistarved rats may have resulted from a decrease in muscle components with an affinity for cesium (for instance, an increase in the relative amount of connective tissue in muscle may have resulted in a decrease in TRI); this would be analogous to the size effect postulated for kidney in mice (10).

Radiation hazard to an organ or an animal is determined by the amount of isotope present and the total time period during which the isotope is in the pertinent tissue. Thus, the presence of a small amount of isotope over an extended period may represent the major biological hazard of a radionuclide. Future studies involving diet modification may be a useful method for reducing radiation hazard from internal emitters. This study of ^{137}Cs metabolism demonstrates the susceptibility of radioisotope metabolites to diet composition, and the treatment, special diet, can be administered continuously over extended periods of time.

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Effects of Two Synthetic Antioxidants, Vitamin E, and Ascorbic Acid on the Choline-deficient Rat [1,2]

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ABSTRACT The purpose of this work was to determine the effects of two synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), *dl*-α-tocopherol and ascorbic acid on the fatty liver and on the renal and cardiovascular systems of choline-deficient rats. Weanling and 6-week-old male rats were fed casein-peanut meal diets devoid of choline for 8 and 10 days, respectively. Survivors were fasted and then killed, and tissues collected for serum and liver lipid analyses and for morphologic assessment of tissue damage. Fatty liver, hemorrhagic kidneys and cardiovascular damage characteristic of choline deficiency were observed in deficient animals of both ages. However, BHA and BHT protected rats in both age groups from heart and aorta damage and also prevented renal damage in the 6-week-old group. Mortality was decreased by tocopherol, ascorbate and BHA and BHT, but most notably by the latter two. In weanling rats all additives increased serum lipids and all except ascorbate decreased liver lipids. In 6-week-old rats serum lipids varied, whereas liver lipids were increased by tocopherol and ascorbate and decreased by BHA and BHT. We conclude that tocopherol and BHA and BHT tend to decrease the effects of choline deficiency on the liver, renal and cardiovascular systems whereas ascorbate enhances them. Possible mechanisms for the various effects are discussed.

The most common response of the liver to metabolic injury is an accumulation of lipid, and the profound increase in the hepatic lipid content of choline-deficient animals has led many investigators to use this system as a model for the study of lipid metabolism. Despite the numerous investigations concerned with the fatty liver of choline deficiency and the many hypotheses proposed to explain it in precise biochemical terms, the pathogenetic basis for this anomaly has not been completely elucidated. There is now convincing evidence for a block in the secretion of triglycerides from the liver into the plasma of choline-deficient rats, but the exact mechnisms underlying the fatty liver and the interrelationship of lipid accumulation and the renal and cardiovascular lesions are largely unexplained (1).

The interesting reports of Porta and Hartroft (2), DiLuzio (3) and Monserrat and Porta [3] concerning the protective effects of antioxidants on fatty liver and tissue damage prompted us to examine the influence of some natural and synthetic antioxidants on the tissues of choline-deficient rats. Ascorbic acid was also included because we have observed a profound enhancement of copper-deficiency vascular lesions by this vitamin [4] and wanted to determine whether it had a similar effect on the lesions of choline-deficient rats. This report describes the effects of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), *dl*-α-tocopherol, ascorbic acid and a combination of the latter two on serum and liver lipids and on renal and cardiovascular lesions in the choline-deficient rat.

MATERIALS AND METHODS

Male rats of the Charles River (CD) strain of two ages were used in these studies. Two experiments were conducted with 6-week-old rats that weighed about 140 g

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[1] Supported in part by National Institutes of Health Grant no. IR-IHE 10112.

[2] This manuscript is contribution no. 1348 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

[3] Monserrat, A. J., and E. A. Porta 1968 Effects of *N,N'*-diphenyl-*p*-phenylenediamine on the kidney of choline-deficient weanling rats. Federation Proc., 27: 409 (abstract).

[4] Hunt, C. E., J. M. Landesman and P. M. Newberne 1967 Copper deficiency and ascorbic acid interrelationships in chicks. Federation Proc., 26: 633 (abstract).

when placed on the experimental diets. Fifteen rats were used in each of six dietary treatments per experiment for a total of 180 animals. Because of their age and relative resistance to death from hemorrhagic kidneys, they were considered marginally deficient in choline and were fed their respective diets for 10 days. Three-week-old weanling rats were used in the other experiments; they weighed about 45 g at the start of each experiment and were used to study the effects of the dietary additives on acute choline deficiency. Because of their marked sensitivity to the deficiency they were kept on the experiment for only 8 days. Three experiments were conducted with the weanling rats; 15 were used in each of six dietary treatments per experiment for a total of 270 animals. All animals were housed in single, screen-bottom cages in air-conditioned quarters, and experimental groups given dietary additives were pair-fed to the deficient groups. Results are reported for the combined trials in each of the two age groups. The composition of the basal diet and the quantities of the dietary additives are given in table 1. Although the casein-peanut meal diet used in these studies results in a good rate of growth in rats, it is marginal in methionine content (about 0.3%) and, therefore, permits accurate measurement and control of lipotrope intake by the animals. Beef fat was used as the source of lipid to enhance the effects of choline deficiency (4); vitamin B_{12} was deleted from the deficiency diet to minimize de novo synthesis of choline (5, 6).

The rats were fasted for 18 hours before they were exsanguinated under light ether anesthesia. Serum was separated, frozen and stored for lipid analysis at a later time. Livers were weighed and samples of the left, median and right lobes taken for histologic examination; the remainder was frozen for lipid analysis and for histochemical demonstration of lipid. Kidneys were weighed and prepared, along with heart and aorta, for routine histologic study using standard paraffin techniques (4). Sections were cut on a freezing microtome and stained with oil red O to demonstrate lipid. Alcian blue-periodic acid Schiff (PAS) stain on paraffin sections was used to demonstrate mucopolysaccharides.

Liver and kidney lesions were graded from zero to 4, according to severity, and averaged. A small amount of stainable liver lipid was graded 1 + and increments were graded accordingly, up to 4 +, which indicated that the entire lobule from the central vein to the portal area was saturated. Renal tubule swelling and necrosis with casts, myocardial and aortic lipid and necrosis were all graded in a similar manner. The incidence of lesions of the heart and aorta is represented in the tables as the number affected over the total number of animals in the group.

Total lipids of serum and liver were extracted with boiling chloroform and redistilled methanol (2:1) in a micro-Soxhlet extraction apparatus (7) and measured gravimetrically. They were then dissolved in chloroform, and aliquots taken for assay of total cholesterol by the Lieberman-Burchard reaction and of total lipid phosphorus by the Fiske-Subbarow method (7, 8).

TABLE 1

Composition of the diets

Basal diet		Additives	
	g/kg		*per g of diet*
Casein, alcohol extracted	60	*dl*-α-tocopherol	0.4 IU
Peanut meal, alcohol extracted	250	Ascorbic acid	5.0 mg
Sucrose	270	BHA	1.0 mg
Vitamin mix [1]	20	BHT	1.0 mg
Salt mix [2]	50	Choline	3.0 mg
Beef fat	340	Vitamin B_{12}	0.05 μg
Corn oil	10		

[1] Newberne, P. M. 1962 The subcommissural organ of the vitamin B_{12}-deficient rat. J. Nutr., 76: 393.

[2] Hegsted, D. M., R. C. Miller, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

RESULTS

Six-week-old rats: marginal choline deficiency. Rats placed on the experimental diets at 6 weeks of age exhibited symptoms and lesions characteristic of a mild choline deficiency after 10 days on the diet (tables 2 and 3). There was no mortality during the experimental period in any of the 6-week-old groups and, except for the slight depression in body weight in rats treated with BHA and BHT, weight gains were comparable throughout the two experiments (table 2). The absolute and relative weights of the liver were increased in all deficient rats. Dietary supplements of BHA and BHT notably decreased the incidence of liver fat. Ascorbate appeared to decrease it in histologic sections; however, chemical analyses did not agree with the histochemical assessment (table 3), a fact that we are unable to explain at this point. This illustrates that histochemical techniques are not quantitative and serve primarily to identify the location of lipid within the lobule. The other treatments tended to increase slightly the amount of liver lipid. Renal and cardiac lesions were minimal in animals of this age group, but lesions of the aorta occurred in all of the choline-deficient groups except those receiving BHA and BHT.

Serum and liver lipid concentrations for the 6-week-old rats are shown in table 4. Compared with controls, and typical of choline deficiency, serum total lipids were lower and liver total lipids higher in the deficient rats. Liver cholesterol content was also somewhat increased by choline deficiency, but liver phospholipid content was decreased. Ascorbic acid aggravated the deficiency by increasing the concentration of fat and decreasing the concentration of phospholipid in the liver. The group supplemented with BHA and BHT had the lowest level of liver total lipids and the highest level of serum total lipids of any of the choline-deficient groups, although their concentration of liver fat, including cholesterol, was elevated with respect to the choline-supplemented control; liver phospholipid values approximated those of the controls.

Weanling rats: acute choline deficiency. The weanling rats were fed their respec-

TABLE 2

Body and liver weights — 6-week-old rats [1]

Treatment	Body wt	Liver wt
	g	*g*
Control	189.5 ± 7.0	5.75 ± 0.2
Choline deficiency	188.5 ± 10.8	7.21 ± 0.4
Choline deficiency + tocopherol	187.5 ± 8.2	6.93 ± 0.3
Choline deficiency + ascorbate	191.9 ± 12.0	7.22 ± 0.5
Choline deficiency + tocopherol + ascorbate	193.2 ± 7.5	7.47 ± 0.4
Choline deficiency + BHA and BHT	177.2 ± 8.4	6.64 ± 0.4

[1] Values are given as the means ± SE.

TABLE 3

Effects of antioxidants on mortality and lesions in choline-deficient 6-week-old rats

Treatment	10-day mortality [1]	Histologic lesions			
		Liver [2]	Kidney [2]	Heart [1]	Aorta [1]
Control	0/30	0.0	0.0	0/30	0/30
Choline deficiency	0/30	2.0	0.1	3/30	6/30
Choline deficiency + tocopherol	0/30	2.4	0.2	0/30	3/30
Choline deficiency + ascorbate	0/30	1.3	0.0	0/30	9/30
Choline deficiency + tocopherol + ascorbate	0/30	2.5	0.2	3/30	6/30
Choline deficiency + BHA and BHT	0/30	0.4	0.0	0/30	0/30

[1] Number of animals affected over number in group.
[2] Graded on an arbitrary basis according to the severity of the lesion and expressed as means. Stainable liver lipid was graded 1+ for a small amount about the centrilobular vein; increased amounts were graded accordingly to 4+, which was equated with virtual saturation of the entire lobule. Renal tubule necrosis and myocardial and aortic medial necrosis were graded in a similar manner.

TABLE 4

Serum and liver lipids in 6-week-old rats [1]

Treatment	Total lipids		Total cholesterol		Total phospholipids	
	Serum	Liver	Serum	Liver	Serum	Liver
	mg/100 ml	*g/100 g dry wt*	*mg/100 ml*	*g/100 g dry wt*	*mg/100 ml*	*g/100 g dry wt*
Control	728* ± 12	28* ± 3	150* ± 10	1.43* ± 0.08	102* ± 12	11.6* ± 0.08
Choline deficiency	465 ± 10	49 ± 4	110 ± 8	1.68 ± 0.10	84 ± 4	8.4 ± 0.09
Choline deficiency + tocopherol	330* ± 12	51 ± 4	132* ± 10	1.14* ± 0.07	70* ± 4	7.7* ± 0.2
Choline deficiency + ascorbate	543* ± 8	56* ± 3	180* ± 9	1.50* ± 0.09	88 ± 5	7.3* ± 0.06
Choline deficiency + tocopherol + ascorbate	528* ± 16	58* ± 4	148* ± 7	1.34* ± 0.05	83 ± 4	6.7* ± 0.6
Choline deficiency + BHA and BHT	691* ± 12	45 ± 5	156* ± 6	1.74 ± 0.06	97* ± 6	10.0* ± 0.4

[1] Values are given as the means ± SE.
* Difference from choline-deficient rats significant ($P < 0.05$, Student's t test).

TABLE 5

Body and organ weights — weanling rats [1]

Treatment	Body wt	Liver wt	Kidney wt
	g	*g*	*g*
Control	79.8 ± 5.0	2.97 ± 0.28	0.87 ± 0.17
Choline deficiency	59.3 ± 3.8	3.23 ± 0.37	1.22 ± 0.38
Choline deficiency + tocopherol	58.6 ± 2.6	3.46 ± 0.35	1.21 ± 0.25
Choline deficiency + ascorbate	64.6 ± 3.0	3.78 ± 0.60	1.24 ± 0.23
Choline deficiency + tocopherol + ascorbate	64.2 ± 2.9	3.56 ± 0.52	1.06 ± 0.18
Choline deficiency + BHA and BHT	62.7 ± 4.0	3.52 ± 0.47	0.91 ± 0.16

[1] Values are given as the means ± SE.

TABLE 6

Effects of antioxidants on mortality and lesions in choline-deficient weanling rats

Treatment	8-day mortality [1]	Histologic lesions			
		Liver [2]	Kidney [2]	Heart [1]	Aorta [1]
Control	0/45	0.0	0.0	0/45	0/45
Choline deficiency	24/45	3.0	2.3	15/45	6/45
Choline deficiency + tocopherol	18/45	2.6	2.6	3/45	9/45
Choline deficiency + ascorbate	18/45	2.2	2.6	9/45	15/45
Choline deficiency + tocopherol + ascorbate	18/45	2.5	2.4	12/45	3/45
Choline deficiency + BHA and BHT	9/45	1.8	1.0	0/45	0/45

[1] Number of animals affected over number in group.
[2] Graded on an arbitrary basis (as described in table 3, footnote 2) according to the severity of the lesion and expressed as means.

tive diets for 8 days, during which time many of them succumbed with symptoms and lesions of severe choline deficiency. Body and liver weights were comparable for all deficient groups (table 5). Supplements of tocopherol plus ascorbate resulted in diminished renal weights but the most pronounced effect was in the BHA and BHT-supplemented group. In general, renal weights paralleled the incidence and severity of renal damage (table 6). Mortality varied among the deficiency groups,

TABLE 7

Serum and liver lipids in weanling rats [1]

Treatment	Total lipids		Total cholesterol		Total phospholipids	
	Serum	Liver	Serum	Liver	Serum	Liver
	mg/100 ml	*g/100 g dry wt*	*mg/100 ml*	*g/100 g dry wt*	*mg/100 ml*	*g/100 g dry wt*
Control	805* ±8	30* ±1	168* ±4	1.40* ±0.07	133* ±5	10.5* ±0.4
Choline deficiency	593 ±9	54* ±2	121 ±10	1.20 ±0.05	171 ±4	6.5 ±0.6
Choline deficiency + tocopherol	843* ±5	48* ±1	100* ±3	1.25 ±0.06	171 ±4	8.1* ±0.3
Choline deficiency + ascorbate	771* ±11	59* ±2	140* ±6	1.62* ±0.11	139* ±9	5.9 ±0.8
Choline deficiency + tocopherol + ascorbate	849* ±12	46* ±4	138* ±3	1.28 ±0.04	158* ±7	7.4* ±0.4
Choline deficiency + BHA and BHT	721* ±10	43* ±7	150* ±7	1.55* ±0.08	139* ±6	9.4* ±0.2

[1] Values are given as the means ± SE.
* Difference from choline-deficient rats significant ($P < 0.05$, Student's t test).

but the greatest number of deaths occurred in the group fed the choline-deficient diet with no additives, and the lowest mortality occurred in the group fed the choline-deficient diet supplemented with BHA and BHT (table 6). Histochemical localization of lipids in the liver paralleled that observed in the 6-week-old rats except that ascorbate did not tend to lower the amount of lipid visualized in weanling animals. Liver fat and kidney lesions were observed in all the choline-deficient groups, but the lesions in the BHA and BHT-supplemented group were the least severe. Cardiovascular damage was observed in all the choline-deficient groups except those supplemented with BHA and BHT. Of the remainder of the groups, it is considered noteworthy that the choline-deficient, vitamin E-supplemented group demonstrated the lowest number of heart lesions and the choline-deficient, ascorbic acid-treated group demonstrated the highest incidence of aortic lesions.

Table 7 lists the results of analyses for serum and liver lipids in the weanling groups of rats. Compared with the choline-supplemented controls, choline deficiency characteristically lowered the serum total lipids and elevated the liver total lipids. In contrast to the 6-week-old rats, in weanling rats liver cholesterol was not elevated due to choline deficiency and the level of liver phospholipids was decreased.

In agreement with data from the 6-week-old rats, ascorbic acid aggravated choline deficiency in the weanling rats; the concentration of liver fat was increased and that of the liver phospholipids decreased. Supplements of tocopherol, however, increased serum total lipids and decreased liver fat; these changes were somewhat similar to those observed after choline supplementation, and under these conditions the concentration of liver phospholipids increased to a level comparable to the choline-supplemented controls. The BHA and BHT-supplemented group exhibited the lowest liver fat of any of the choline-deficient groups, but liver total fat and cholesterol in this group did not approximate that of the choline-supplemented controls. The liver phospholipid concentration of the BHA and BHT-supplemented group approximated that of the control animals.

Histology. The various lesions of the organs and tissues were of the same nature in all groups of deficient animals, irrespective of supplements, and varied only in degree of severity. Thus, only representative lesions will be described and illustrated. The authors are aware of the many descriptions and illustrations of gross and microscopic lesions of choline deficiency in the literature but feel that this additional grouping of illustrations of liver, cardiovascular and renal pathology is justi-

fied because of revised concepts of the pathogenesis of some of them. Figure 1 illustrates the gross appearance of the enlarged, pale, fatty liver of choline deficiency familiar to investigators working with this system. Note the bulging of the surface, a result of the accumulation of large quantities of lipid in distended single and coalescing lipid-laden parenchymal cells. Figure 2 shows the microscopic appearance of the large amount of lipid accumulated in the liver parenchymal cells of a choline-deficient rat. The amount of stainable lipid was decreased by supplementing the choline-deficient diet with BHA and BHT (fig. 3). In figure 3 the centrilobular localization of lipid characteristic of choline deficiency can be seen.

Hemorrhagic kidneys, a cardinal lesion of choline deficiency in the weanling rat, are shown in the upper portion of figure 4. Just prior to hemorrhage there is leakage of large quantities of protein into the renal tubules (fig. 5), a factor that must be taken into account when one attempts to evaluate results of biochemical analyses on such organs. The large amount of protein in the tubules could radically alter the protein-nucleic acid relationships calculated from chemical analysis but fail to indicate the basic alteration in parenchymal cells. Figure 6 illustrates the severe damage to the renal tubule epithelium attendant upon choline-deficiency syndrome. Note the enlarged hypertrophic and degenerate epithelial cells, many of which are shed into the lumen and are often found in close association with the protein casts in the tubules.

The hypothesis that renal hemorrhage in choline deficiency results from large accumulations of lipid in the tubule epithelium and consequent compression and rupture of interstitial capillaries has now been abandoned as untenable.[5] Figure 7 illustrates the relatively small amount of stainable lipid at the base of the tubule epithelial cells of the choline-deficient rat. The amount may vary considerably among a group of deficient animals but it does not appear to parallel the incidence of hemorrhage. Of much greater consequence, however, is the remarkable increase in tubule cell hyperplasia, which is illustrated by the two mitotic figures shown in figure 7. This phenomenon, as well as the large amount of protein leaked into the tubules, no doubt contributes to the increased size and weight of the kidneys of choline-deficient rats. We feel that these are the basic alterations in the choline-deficient kidney whether or not frank hemorrhage occurs, and we cannot overemphasize the need to determine accurately the morphologic status of an organ before attempting to evaluate data derived from biochemical analyses. One can readily recognize that the chemical analysis of a kidney such as the one illustrated in figure 5 will differ greatly from determinations derived from one without the large protein cast component. Furthermore, the erythrocytes residing in a hemorrhagic kidney will influence appreciably analytical data derived from such an organ. Thus, when rat organs are analyzed, pooling organs, such as hemorrhagic with nonhemorrhagic kidneys or degenerate with nonaffected hearts, will dilute and probably mask important biochemical changes or at least bias the interpretation of the results.

Figure 8 illustrates the accumulation of stainable lipid in a coronary artery of a choline-deficient rat, and the typical focal accumulation of lipid in the myocardium of the deficient rat is shown in figure 9. A concomitant to the increased lipid in these tissues is an accumulation of acidic and neutral mucopolysaccharides, illustrated in figure 10. The well-known vascular damage of choline deficiency appears to be initiated by an influx of lipid which accumulates in the smooth muscle cells (fig. 11). This is accompanied by swelling, fragmentation, and lysis of the elastic lamellae and early degeneration of interlamellar muscle and connective tissue cells (fig. 12). Concomitant to these changes there is an accumulation of acid mucopolysaccharides in the interlamellar spaces as the degenerative alterations progress (fig. 13). The sequence of events which lead to cardiovascular damage in the choline-deficient rat therefore appears to be initiated by a deposition of lipid in tissues. Concomitant to or shortly after lipid deposition there are early degenerative changes in parenchymal cells and derangement of mucopolysaccharides. It is significant that the synthetic antioxidants BHA and BHT

[5] Hartroft, W. S. 1967 personal communication.

and, to some extent, tocopherol tend to inhibit the initiation of such lesions. Of interest and perhaps of even greater significance is the observation that ascorbate tends to enhance and intensify choline-deficiency tissue damage.

DISCUSSION

The biochemical mechanisms whereby choline deficiency induces fatty liver and renal and cardiovascular damage in rats are not presently well understood despite the large amount of effort that has gone into researching the problem. Available evidence suggests that the lack of sufficient choline for lecithin synthesis results in a decreased amount or reduced turnover of phospholipids, particularly those to be incorporated into cell membranes. This may then result in a shift in favor of the direct synthesis of lecithin from diglyceride and CDP-choline (9) at the expense of the indirect pathway, the stepwise methylation of phosphatidyl ethanolamine (10, 11), which in turn is derived from phosphatidyl serine (12–14). Fatty acid studies have indicated that this shift may have a direct bearing on the types of phospholipids synthesized; for example, it appears that in choline-deficient rats, the arachidonic acid content of the serum and tissue phospholipids decreases whereas it increases in serum and tissue triglycerides (13–15). Furthermore, most of the increase in liver lipids in choline deficiency is in the triglyceride fraction. It is also of interest to note that arachidonate-containing phospholipids have been associated primarily with the indirect pathway for lecithin synthesis whereas palmitate- and linoleate-containing phospholipids have been associated primarily with the direct pathway (13). In this regard Tinoco et al. (13) reported that the decrease in serum phospholipid arachidonic acid was accompanied by an increase in serum phospholipid linoleic acid, and the increase in serum triglyceride arachidonic acid was accompanied by a decrease in oleate in this fraction.

The generalized hypolipidemia induced by choline deficiency has been associated with a preferential decrease in the hepatic output of longer chains and more unsaturated fatty acids (13). The defect could be due to an impairment of indirect synthesis of lecithin, resulting first in a decreased transfer of the more unsaturated fatty acids (from the 2-position of lecithin) to cholesterol, and then in a decrease of plasma-esterified cholesterol (13). Norkin (14) suggests that this defect may explain his observation of a higher rate of accumulation of the longer-chain and more unsaturated fatty acids in hepatic triglycerides of choline-deficient rats, which occurred at the expense of shorter-chain and more saturated fatty acids.

The membranes of the rat liver endoplasmic reticulum contain high levels of arachidonic acid in the phospholipids (16), and choline deficiency appears to cause an increased requirement for arachidonate-containing phospholipids.[6] Thus, there exists the possibility that arachidonate-containing phospholipids may have an important bearing on the integrity of membranes, such as that of the endoplasmic reticulum, and on the synthesis of transport lipoprotein; however, these factors require experimental confirmation.

An increase in lipid peroxide formation has been indicated by diene conjugation determinations in choline deficiency-induced hemorrhagic kidneys,[7] and the possible damaging influence of lipid peroxidation on lysosomal membranes has been reported (17). Thus, the effects of antioxidants on the choline-deficiency syndrome may be mediated through their effect on lipid peroxidation. Monserrat et al. (18) have reported that the earliest detectable changes in the kidneys of choline-deficient weanling rats were dilatation of the endoplasmic reticulum and lysosomal changes. An increase in the number and size of lysosomes was followed by coalescence and membrane disruption. The reason for the lysosomal proliferation is not known nor is there an explanation for the rupture but it is suggested that the disruption of membranes may have a direct bearing on the onset of necrosis. It is further suggested that the mechanisms leading to the choline deficiency-induced alter-

[6] Barker, M. O., and J. G. Hamilton 1968 Incorporation of ^{14}C-methyl groups from methionine into subfractions of rat liver phosphatidyl choline. Federation Proc., 27: 361 (abstract).

[7] See footnote 3.

ations in liver and kidney may involve quite different metabolic pathways.

Our results indicate that the synthetic antioxidants, BHA and BHT, are able to protect the choline-deficient kidney against the formation of lesions detectable by light microscopy; this is in agreement with the report of a similar renal protective action by the antioxidant diphenylparaphenylenediamine (DPPD).[8] The partial alleviation of the choline deficiency-induced fatty liver by BHA and BHT is not understood, but it is of interest that ascorbic acid, which has been shown to enhance peroxidation in vitro (16, 19, 20), may have aggravated the choline deficiency-induced fatty liver in addition to increasing the severity of the aortic lesions. Furthermore, a profound effect of these antioxidants was evident for the cardiovascular system since lesions of the aorta and heart were completely prevented by BHA and BHT.

Our results also indicate that in weanling rats tocopherol decreased the accumulation of the liver fat associated with choline deficiency, and exerted a protective effect on the heart but not on the aorta. It is noteworthy that vitamin E has been found in rather high concentrations in heart muscle (21).

Although the mechanisms involved in the pathogenesis of the various choline deficiency-induced lesions are complex, lipid peroxidation may be one of the factors involved; however, the membrane-stabilizing effect of the antioxidants and their role in maintaining membrane integrity require further study.

Finally, we wish to emphasize the critical need to correlate the morphologic changes in the organs and tissues of choline-deficient rats with biochemical analyses. Lesions detectable by light microscopy must surely result in biochemical values different from those found in an organ or tissue exhibiting no morphologic change or, at most, only ultrastructural changes. The more severe lesions such as the hemorrhagic kidney may well mask the important alterations in metabolism which occur at an earlier stage and which actually represent the basic significant change. Although the liver is perhaps not as critical in this regard as the renal and cardiovascular tissues, supporting morphologic evidence would permit greater confidence in biochemical parameters.

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PLATE 1

EXPLANATION OF FIGURES

1 Gross photograph of a fatty liver typical of acute choline deficiency.

2 Section of liver illustrating lesions typical of choline-deficient rats after 7 days on the ascorbic acid-supplemented diet. The section was cut on a freezing microtome and stained with oil red O. The centrilobular vein in the center is surrounded by parenchymal cells distended with lipid. Oil red O. × 80.

3 Section of liver illustrating lesions typical of choline-deficient rats given the diet supplemented with BHA and BHT for 7 days. The section was treated the same as that shown in figure 2. Note the reduction in the amount of stainable lipid, which correlates with chemical lipid analysis on livers from the same group (table 7). Oil red O. × 70.

4 Hemorrhagic kidneys of a choline-deficient rat (above, white arrows) compared with the kidneys of a control animal (below, black arrows). The large increase in size is due primarily to an increase in parenchymal cell proliferation and a small amount of lipid. Terminally, there is some increase in size due to hemorrhage, but this accounts for only a small part of the total increase in the size of the organ.

5 Corticomedullary region of a kidney from a choline-deficient rat at the prehemorrhagic stage. Note the large amount of cast formation made up primarily of serum proteins which have leaked through the glomerulus. The large amount of tubular material must be taken into account in chemical determinations on prehemorrhagic and hemorrhagic kidneys. PAS. × 48.

6 High magnification of a section of the kidney from figure 5 illustrating the PAS-positive material in the tubules and the swelling and sloughing of the tubule epithelium. PAS. × 420.


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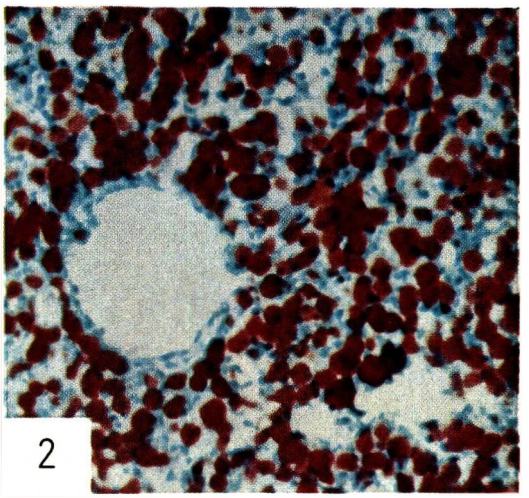
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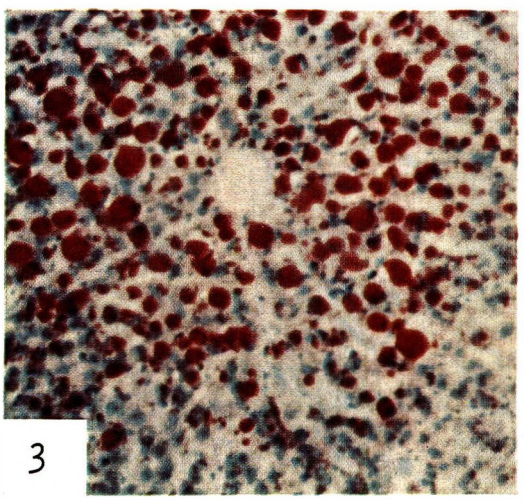
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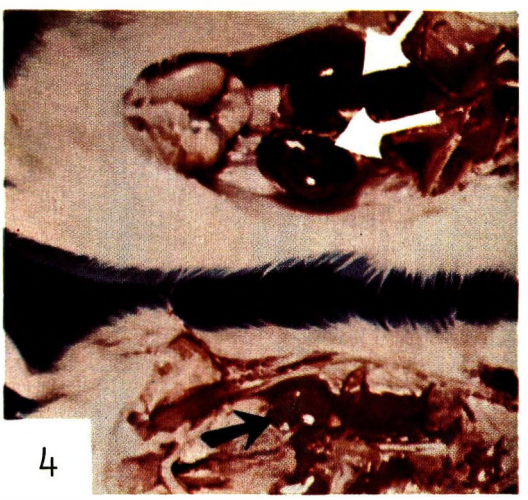
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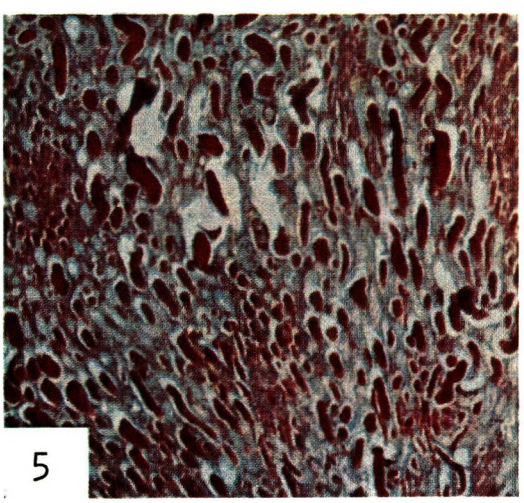
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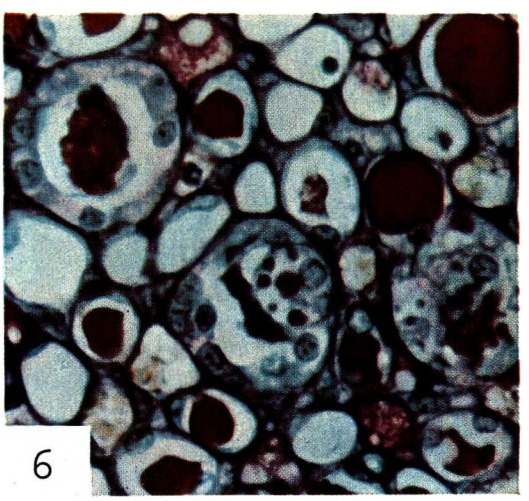
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PLATE 2

EXPLANATION OF FIGURES

7 Kidney tubule from a choline-deficient rat. Frozen section stained with oil red O for lipid illustrates the relatively small amount of lipid in the tubule epithelium (white arrows) and two mitotic figures (black arrows), one of which contains lipid. Oil red O. × 420.

8 Coronary artery from a choline-deficient rat. Note the lipid accumulation in the wall of the vessel. This precedes the degenerative alterations observed at a slightly later period. Oil red O. × 420.

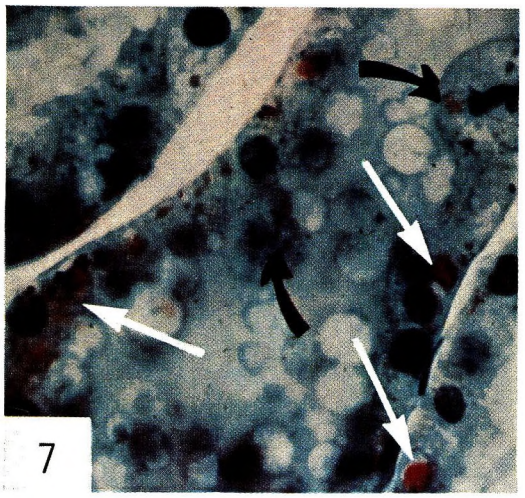
9 An area of focal myocarditis in the heart of a choline-deficient rat. Lipid accumulation is evident in muscle cells, and reactive cells have infiltrated the interstitium. Oil red O. × 90.

10 Cross-section of the myocardium in an area of focal degeneration similar to that shown in figure 9. Pale green and blue staining material is acid (sulfated) mucopolysaccharides, pink staining material is degenerating myocardial fiber and yellow staining material is residual intact myocardial fiber. Alcian blue-PAS. × 390.

11 Longitudinal section of aorta from a choline-deficient rat. Note the lipid accumulation in the smooth muscle cells of the media. Oil red O. × 420.

12 An early plaque in the media of the aorta from a choline-deficient rat. The elastic fibers are swollen and have undergone fragmentation. Degenerative changes of interlaminar muscle appear as greenish-yellow material between two degenerate elastic lamellae. Verhoeff's. × 420.

13 Longitudinal section of aorta from a choline-deficient rat. Note that the acid mucopolysaccharides have accumulated in the same areas where lipid is identified in figure 11 and where there is early necrosis, as shown in figure 12. Such areas progress to large plaques involving the entire thickness of the vessel wall if the animal survives for a longer period of time. Alcian blue-PAS. × 420.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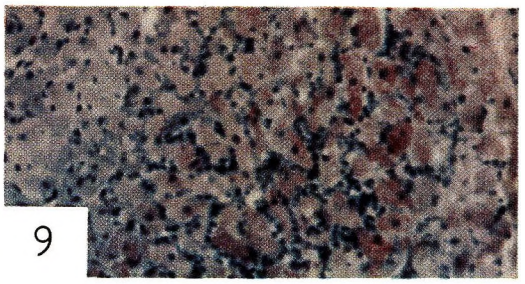
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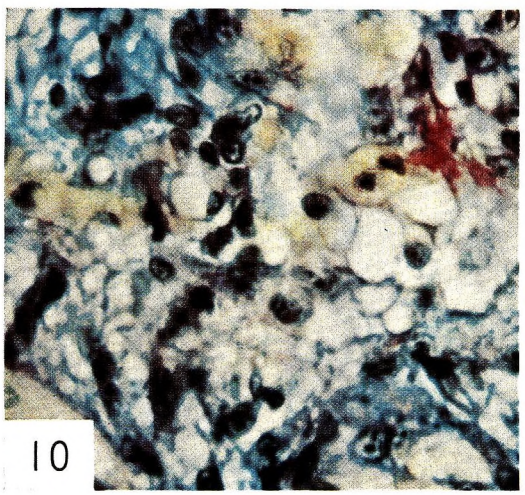
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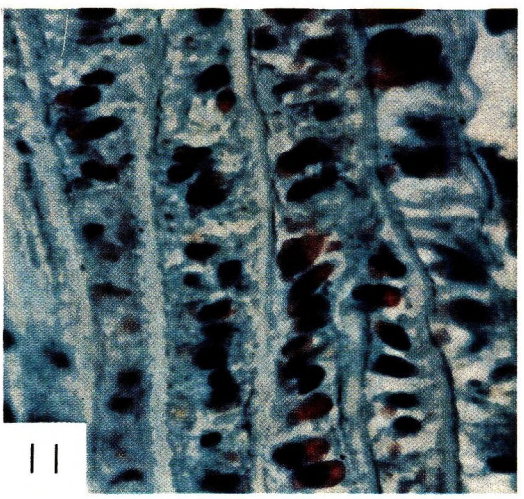
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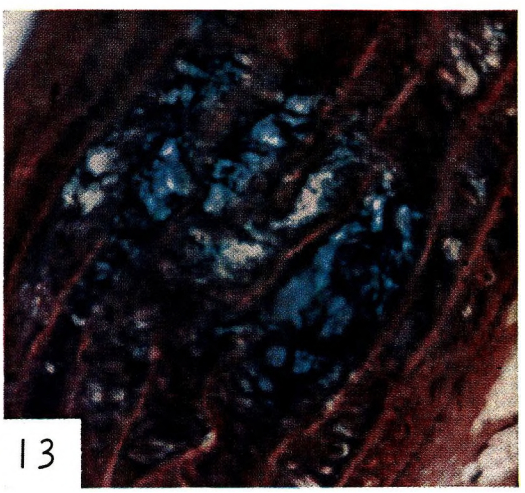
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Status of the Microcirculation During Acute Choline Deficiency [1,2]

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ABSTRACT Weanling 21-day-old male rats of the Fischer strain were fed a choline-deficient diet, some with and some without choline supplementation. Five days later, none had any signs of nephropathy by blood urea nitrogen concentration or by gross and microscopic examination of the kidneys. The mesoappendiceal circulation of the nonsupplemented rats appeared ischemic when compared with that of the choline-supplemented rats. There was a striking alteration in the characteristic reaction of the microvessels to topical epinephrine. In choline-supplemented rats, an average of 0.48 μg/ml epinephrine caused maximal responses of the precapillary sphincters, lesser responses of the precapillary arterioles, and no reaction of the supplying artery. In marked contrast, less than 10% this concentration, 0.03 μg/ml epinephrine, resulted in pronounced vasoconstriction of the supplying artery with minimal or no reactions of the smaller vessels in the rats not supplemented with choline. These data are consistent with our view that the feeding of diets low in choline results in an imbalance in vasoactive mediators due to a decrease in tissue acetylcholine which leads to vasospasm and ischemia of the kidneys resulting in the characteristic nephropathy of acute choline deficiency.

The renal and hepatic manifestations that occur after feeding weanling rats diets low in choline have been known for more than 30 years. Accumulation of liver fat appears to be due to the impaired mobilization of fat from the liver resulting from the low availability of methyl donor compounds (1). The mechanism for the development of the bilateral renal cortical hemorrhagic necrosis, which appears by 6 to 8 days with its biochemical and physiological counterparts, is poorly understood.

Hartroft (2) attributed the development of the renal disease to compression of the cortical capillary plexus by the accumulation of fat droplets in tubular epithelial cells causing tubular swelling; Wolbach and Bessey (3) postulated a cholinergic mechanism in the development of the nephropathy.

To test the latter theory, we set out to explore the possibility that the feeding of diets low in choline resulted in a decrease in tissue levels of acetylcholine which, subsequently, because of the neurohumoral imbalance, led to vasospasm via increased reactivity to pressor amines, renal ischemia, necrosis and hemorrhage. We have previously reported our observations regarding the changes in acetylcholine levels of certain tissues in open-animal-room and germfree rats fed choline-deficient diets (4,5). This paper is concerned with changes in the microcirculation of the mesoappendices of rats fed a choline-deficient diet.

MATERIALS AND METHODS

The diet used and the experimental design have been detailed in other publications (6,7). The diet was adapted from Salmon and Newberne (7), and its content is given in table 1; to increase the severity of the deficiency, the diet was

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TABLE 1

Composition of choline-deficient diet [1]

	g/1000 g diet
Casein, vitamin-free test	57
Peanut meal (double alcohol-water extracted)	238
Sucrose	400
Lard (kettle rendered)	180
Cod liver oil	9.5
Cholesterol	50
Cystine	4
$CaHPO_4 \cdot 2H_2O$	30
KCl	5.7
$MgSO_4$	3.8
$NaHCO_3$	6.6
Ferric citrate	1.16
$MnSO_4 \cdot H_2O$	0.10
$ZnCO_3$	0.10
$CuSO_4$	0.04
KI	0.03
Riboflavin	0.60
Thiamine·HCl	0.30
Calcium pantothenate	0.60
Pyridoxine·HCl	0.30
Niacin	6.00
Menadione	0.15
Inositol	6.00

[1] Salmon and Newberne (7) diet modified by addition of cholesterol and cystine.

modified in our laboratory by the addition of 0.4 g cystine and 5 g cholesterol/100 g diet.

Weanling 21-day-old male rats of the Fischer strain were divided into two groups of equivalent paired weights. All rats were fed the choline-deficient diet ad libitum; one group was given choline chloride [6] in its drinking water in a concentration of 1.5 mg/ml. The other group received no supplement. Both groups drank 10 to 15 ml liquid/day; thus, the choline-supplemented groups had an intake of 15 to 22.5 mg choline chloride/day. On the morning of day 5, the rats were bled (0.1 ml) via retroorbital puncture, and the concentration of blood urea nitrogen (BUN) was determined from a 1:20 sulfuric acid–tungstate protein-free filtrate (8).

The rats were then lightly anesthetized with pentobarbital sodium (2.5 mg/100 g, intramuscularly). The mesoappendix was exteriorized and mounted on an irrigated microscope stage for observation of the microcirculation. From the moment of exposure and throughout the experiment, the tissue was continuously irrigated with mammalian Ringer solution (37.5°) of the following composition per liter: NaCl, 154.3 mmoles; KCl, 5.63 mmoles; $CaCl_2$, 2.16 mmoles; and gelatin 10 mg, adjusted to pH 7.4 with $NaHCO_3$. After the status of the circulation was observed, topical epinephrine [7] was applied in varying concentrations and the reactivity of the microvessels to such test concentrations was recorded visually and by microphotography, according to methods described in detail elsewhere (9,10). The epinephrine was first diluted in distilled water to a concentration of 100 μg/ml to serve as a stock solution. Further dilution (minimum, 100-fold) was made with the Ringer solution just before topical application for each test. The microvascular event (vasoconstriction) in response to topical stimuli by epinephrine was evident within 15 to 20 seconds. The local reaction did not last more than 40 to 45 seconds, and recovery (to control appearance) was complete by 50 seconds. The reactivity was graded for each of 4 parts of the vascular bed (artery, arteriole, metarteriole and precapillary sphincter) as showing a constriction or not (+ or 0). Test doses of epinephrine were varied from lowest concentration to the highest. Observations regarding constriction of the precapillary spincter, metarteriole and small artery were made. Blood pressure was continuously monitored with a transducer,[8] and a polygraph [9] via an indwelling cannula in a femoral artery. Experiments were conducted so that control (choline-supplemented) and dietary choline-deficient rats were alternately studied.

After the reactivity of the vessels of the mesoappendices was determined, the rats were killed; the kidneys were removed, examined grossly, weighed, bisected and placed in 10% buffered formalin. Microscopic sections were stained with hematoxylin and eosin.

RESULTS

The blood urea nitrogen (BUN) concentrations of all rats with or without choline supplementation were normal, 20 mg/100 ml blood (table 2), as were the weights, gross appearances and histologic

[6] Choline chloride, Fisher Scientific Company, New York, N. Y.
[7] Parke Davis and Company, Teterboro, N. J.
[8] Model p 27 Gb, Statham Instruments, Inc., Los Angeles, Calif.
[9] Model 7, Grass Instrument Company, Quincy, Mass.

TABLE 2

Reactivity of the microvessels [1] of choline-deficient rats [2] to topical epinephrine [3]

Animal no. and supplement	BUN [4]	Mean arterial blood pressure	Epinephrine [5]	Vessel type and reactivity			
				Artery	Arteriole	Met-arteriole	Precapillary sphincters
	mg/100 ml	*mm Hg*	*μg/ml*				
1 Choline	20	104	0.1	0	+	+	+
2 Choline	18	98	0.5	0	+	+	+
3 Choline	23	102	0.2	0	0	+	+
4 Choline	22	100	1.0	0	0	+	+
5 Choline	18	110	0.5	0	+	+	+
6 Choline	19	108	0.2	0	0	+	+
1 None	23	98	0.1	+	0	0	0
2 None	22	100	0.01	+	+	0	0
3 None	19	90	0.02	+	0	0	0
4 None	20	104	0.01	+	0	0	0
5 None	18	110	0.05	+	+	0	0
6 None	18	102	0.02	+	+	0	0

[1] Microvessels of the exteriorized mesoappendices.
[2] Male weanling rats of the Fischer strain fed choline-deficient diet for 5 days; 21 days old at start of experiment.
[3] Epinephrine·HCl, Parke Davis and Company, Teterboro, N. J.
[4] BUN = blood urea nitrogen concentration, mg/100 ml whole blood.
[5] Lowest concentration of epinephrine which resulted in a response of the mesoappendiceal circulatory system.

features of the kidneys. In a previous publication (6), a correlation between the level of BUN and the severity of the nephropathy was demonstrated.

The microcirculation of the mesoappendices of rats fed the choline-deficient diet but supplemented with choline in their drinking water appeared normal on microscopic study. The reactivity of the small artery, arteriole and precapillary sphincters was similar to that of rats fed normal rat diets; the concentration of epinephrine required to cause narrowing or closing increased with the diameter of the vessel. The concentration of epinephrine required to elicit a response varied from animal to animal, the range being 0.1 to 1.0 μg/ml. These concentrations resulted in responses of the precapillary arterioles but yielded no reaction of the supplying small artery in the control rats. The concentration of epinephrine required for narrowing of the small artery supplying the area varied from 2 to 5 μg/ml for specific rats.

In marked contrast, the mesoappendices of rats fed the choline-deficient diet and no choline supplement appeared ischemic; there were fewer open capillaries per unit area. In addition, the reactivity of the microvessels to epinephrine was markedly altered. The first observed response to topical epinephrine was a constriction of the supplying small artery; the concentration of epinephrine required to elicit this response varied from animal to animal, the range being 0.01 to 0.1 μg/ml. Such test concentrations of epinephrine resulted in pronounced vasoconstriction of the supplying small artery with minimal or no reaction of the precapillary arterioles and precapillary sphincters. This concentration of epinephrine was less than 10% that required to elicit a response of the capillary sphincters in choline-supplemented rats and was less than 1% the concentration required to elicit a response in the small artery of choline-supplemented rats. Higher concentrations of epinephrine resulted in greater narrowing of the small artery which was reflected by a lessened flow through the corresponding small vessels of the vascular tree. A titration of response to epinephrine by the precapillary sphincters and metarterioles was, therefore, not possible in choline-deficient rats. Thus, the sensitivity of the small supplying artery to epinephrine was markedly increased (table 2, fig. 1). In the majority of the choline-deficient rats, zones of petechial hemorrhages were observed microscopically during the 25-minute period of observation.

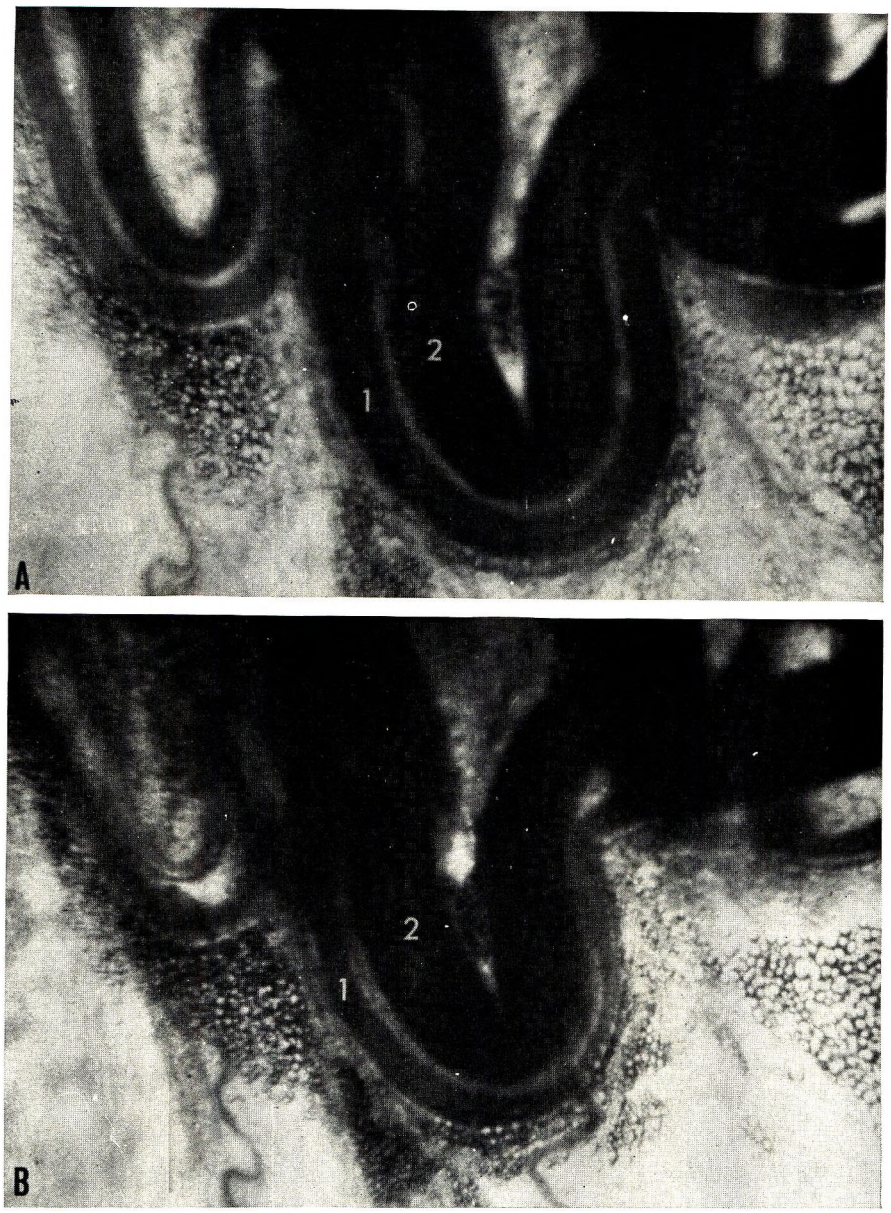

Fig. 1 Microphotograph of the mesenteric circulation showing the artery (1) and vein (2). A: Normal size of artery of rat fed choline-deficient diet prior to the topical application of epinephrine. B: Same artery (1) after topical epinephrine application (0.03 μg/ml). Note approximately 50% narrowing of the artery. × 120.

DISCUSSION

A neurovascular abnormality as a result of acute choline deficiency was postulated by Wolbach and Bessey (3) about 25 years ago. Since that time, several papers have appeared in the literature presenting apparently conflicting data regarding this hypothesis (11–14).

This question was recently explored in our laboratory, and we have published data (4,5) showing, by direct bioassay, decreases in the levels and concentrations

of acetylcholine in the brain, gut and kidneys of choline-deficient rats with no alterations in brain or kidney acetylcholinesterase (gut not assayed for acetylcholinesterase) activity that could account for the changes.

We have shown in the experiments reported in this paper that the mesoappendiceal circulatory beds of animals fed a choline-deficient diet appear ischemic and are markedly sensitive to epinephrine stimulation. These data agree with our theory that a vasoactive material with an opposite effect to epinephrine on vascular smooth muscle, such as acetylcholine, was present in less than normal amounts or activity. In other experiments (10 to 12 days of choline deficiency), we also noted a lessening of peristalsis of the gut of animals fed choline-deficient diets (6), an observation also consistent with decreases in acetylcholine, but no direct measurements of peristalsis were made. We feel that the increased sensitivity of the mesoappendiceal arteries to epinephrine suggests an alteration in the neurohumoral status which is consistent with our previously reported finding of a decrease in acetylcholine concentrations in the kidney, brain and intestine of choline-deficient rats. These data suggest that the mechanism by which choline deficiency results in renal hemorrhagic necrosis is via a generalized imbalance in neurohumoral mediators such that the renal circulation is rendered more susceptible to vasoconstriction by pressor agents. This we think leads to periods of ischemia in the kidney with subsequent necrosis and hemorrhage.

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Serum Cholesterol and Glucose Levels in Rats Fed Refined and Less Refined Sugars and Chromium [1]

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ABSTRACT Refined sugar (sucrose) contains less chromium than partly refined sugar. Because human hypercholesteremia and diabetes mellitus have been believed associated with the consumption of sugar, 200 weanling rats were given a low chromium diet of Torula yeast, lard and sucrose, with essential trace metals in drinking water, to ascertain effects on fasting serum cholesterol and glucose levels. Groups were given white sugar containing 0.02 ppm Cr without and with chromium(III) supplementation (5 ppm in drinking water), less refined "raw" sugar containing 0.06 ppm Cr, and still less refined brown sugar with 0.12 ppm Cr. Serum cholesterol levels were relatively elevated and increased with age in the group receiving white sugar; in that given white sugar plus chromium or brown sugar they were low. Effects were similar in both sexes. Younger rats fed raw sugar had lower levels than those fed white. Fasting serum glucose was relatively low in rats fed brown sugar and in females fed white plus chromium; minimal effects occurred in those given "raw" sugar. These data offer evidence that refined sugar without chromium can relatively elevate serum cholesterol and glucose levels, and that chromium(III) can lower both substances.

The hypothesis that human atherosclerosis is associated with consumption of sugar (sucrose) has been advanced (1). Reports from this laboratory have indicated that rats receiving a standard diet of whole rye flour, dry skim milk and corn oil, fortified by essential trace metals in drinking water, exhibited lower fasting serum cholesterol and glucose levels when soluble trivalent chromium was given than when it was not given (2, 3). Analyses of various sugars showed marked depletion of chromium in refined compared with unrefined samples (4). Therefore, it appeared rewarding to examine the effects on serum cholesterol and glucose levels of rats fed several sugars, using a special diet high in sucrose and low in chromium, and to evaluate effects of added chromium.

METHODS

Rats of the Long-Evans [3] strain were born and reared in a laboratory especially designed to avoid metallic contamination from environmental pollutants (5). Two series of animals were studied. Series 1 consisted of 60 male and 60 female weanling rats divided as litter mates into three groups: Group 1 received refined white sugar; group 2, refined white sugar plus 5 ppm chromic acetate in drinking water; group 3, a relatively unrefined dark brown sugar. They were examined twice, at approximately 5 and 10 to 11 months of age. Series 2 consisted of 40 male and 40 female weanling rats divided as litter mates into two groups: Group 1 received refined white sugar; and group 2, a raw sugar.

The diet was slightly modified from that of Schwarz and Mertz (6), consisting of 50% sucrose, 30% Torula yeast [4] and 15% lard [5] with 5% salt and vitamin mixture (6) added.[6] Calorie equivalents were approximately 26% from protein, 44%

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[3] Random-bred pregnant females were obtained from Blue Spruce Farms, Inc., Altamont, N. Y.

[4] Nutritional Biochemicals Corporation, 26201 Miles Avenue, Cleveland, Ohio 44128.

[5] Pure lard shortening, Tobin Packing Company, Albany, N. Y.

[6] The salt mixture, containing salts of calcium, magnesium, sodium, potassium, iodide, iron and phosphate was identical to that used by Schwarz and Mertz (6); to it was added sodium selenite to provide 0.2 ppm Se, and cystine to provide 0.3% in the diet. The vitamin premix was also identical; to it was added 0.8% vitamins A and D Crystallets. (Abbott Laboratories, Chicago, Ill.)

from sucrose and 30% from fat. Refined white sugar [7] contained 0.11 to 0.21% ash (two samples) and 0.02 to 0.03 μg/g chromium; it was white and free flowing. Brown sugar [8] contained 1.46 to 3.05% ash (two samples) and 0.12 to 0.24 μg/g chromium; it was dark brown in color and sticky, probably from a residue of molasses. The raw sugar [9] contained 0.32% ash and 0.06 μg/g chromium; it was light tan in color, crystalline and free flowing. As wholly unrefined sugar usually contains 3 to 5% ash, this raw sugar was undoubtedly partly refined before shipping from the Philippine Islands, its source. From the ash weight, it was obviously less refined than white and more refined than brown sugar. All sugars came from sugar cane. The white sugar diet contained about 0.1 μg/g chromium in several analyses.

The drinking water given rats contained the following metals as soluble acetates or citrates: (in parts per million) zinc, 50; copper, 5; manganese, 10; cobalt, 1; and molybdenum, 1 (5). Chromium was added to this water at 5 ppm for group 2.

Analyses of serum were made on 48 animals/day. Warmed rats were bled from the tail after fasting for 18 hours. Blood was centrifuged, and serum was analyzed for cholesterol by the method of Huang et al. (7) and for glucose by the glucose oxidase method of Washko and Rice (8), using premixed reagents and a spectrophotometer.[10] Duplicate and replicate analyses agreed within 3% and 4%, respectively. Analyses of sugars were made by atomic absorption spectrophotometry [11] using a Boling burner, after ashing at 450° in muffle furnaces.

Owing to a laboratory misfortune, female rats receiving white sugar and white sugar plus chromium were not bled for analysis at 150 days of age.

RESULTS

Cholesterol. Serum cholesterol levels of male rats in series 1 at two age intervals and series 2 at one age interval are shown in table 1. Young male rats had a lower mean level when receiving brown sugar than white, and a lower level when receiving raw sugar than white. When the same animals were studied at older ages, those on white sugar showed a significantly higher mean level than when younger and a significantly higher level than those given chromium or brown sugar. According to these data, serum cholesterol

[7] Domino brand, American Sugar Company, New York, N. Y.
[8] See footnote 7.
[9] Supplied by John L. Hickson, Ph.D., Sugar Research Foundation, New York, N. Y., from a shipment of Philippine sugar to American Sugar Company, Brooklyn, N. Y. It contained 97.88% sucrose, 0.73% invert sugar, and 0.7% undefined material.
[10] Berkeley Medical Instruments, New England X-Ray and Electronic Equipment, 157 Sutherland Road, Brookline, Mass. 02146.
[11] Model 303, Perkin-Elmer Corporation, Main Avenue, Norwalk, Conn.

TABLE 1

Fasting serum cholesterol levels in rats fed three sugars and chromium

Sugar	Male			Female		
	Age	Serum cholesterol		Age	Serum cholesterol	
	days	*mg/100 ml* [1]	*P* [2]	*days*	*mg/100 ml* [1]	*P* [2]
Series 1						
White	150	74.0 ± 2.90	—	—	—	—
White + Cr	151	67.7 ± 3.62	ns [3]	—	—	—
Brown	157	61.8 ± 4.67	< 0.025	157	69.1 ± 3.11	—
White	324	104.2 ± 6.56 [4]	—	318	109.8 ± 11.40	—
White + Cr	322	57.0 ± 3.47 [5]	< 0.001	330	59.2 ± 4.37	< 0.001
Brown	318	54.9 ± 3.36	< 0.001	322	77.2 ± 2.24 [6]	< 0.005
Series 2						
White	128	60.2 ± 3.21	—	128	59.7 ± 1.54	—
"Raw"	128	53.4 ± 1.73	< 0.05	128	44.0 ± 1.68	< 0.001

[1] Mean ± SEM. Each mean is that of 12 rats randomly selected from each group. Chromium was supplied as indicated at 5 ppm in drinking water.
[2] P is significance of difference of mean from that of white sugar group.
[3] Not significant.
[4] Differs from day 150 value, $P < 0.001$.
[5] Differs from day 151 value, $P < 0.025$.
[6] Differs from day 157 value, $P < 0.025$.

levels of males increased with age when they were fed white sugar, but did not increase when fed brown sugar or when chromium was added to the white sugar. In female rats fed white sugar, mean cholesterol levels were higher than when they were given chromium or brown sugar.

In series 2, although serum cholesterol levels in both sexes fed white sugar at 4 months of age were low, the levels were even lower in those given raw sugar.

When male rats at 128, 150 and 324 days of age fed refined white sugar were considered together, a significant rise in serum cholesterol levels occurred at each age interval ($P < 0.005$). In females a rise occurred from 128 to 318 days ($P < 0.001$). The reverse occurred in males given chromium supplements or brown sugar; cholesterol levels declined with age. In females fed brown sugar, levels increased somewhat with age.

Comparing the sexes at each age interval, significant differences in mean serum cholesterol levels in males and females did not usually occur. Older females fed brown sugar, however, had higher levels than males ($P < 0.001$), and young males fed raw sugar had higher levels than females ($P < 0.001$).

Glucose. Rats of both sexes fed brown sugar had much lower serum glucose levels than did rats fed white sugar without added chromium (table 2). Those given raw sugar had slightly lower levels than did those given white sugar, although all values were elevated. When chromium was added to white sugar, young male rats showed possibly lower glucose levels than when it was not; this questionable effect was not observed in older males. Female rats given chromium supplements, however, had much lower glucose levels than did those given white sugar alone, which did not differ from levels of females given brown sugar.

Considering both series of rats fed white sugar together, levels in males appeared to decline from age 128 to 150 days, but not thereafter ($P < 0.01$); and in females, from age 128 to 318 days ($P < 0.001$).

A comparison of the sexes revealed that glucose levels were higher in females than in males fed brown sugar at 157 days of age ($P < 0.001$), and in those fed white sugar at 128 days ($P \sim 0.025$) and 318 days of age ($P < 0.01$). Levels were lower in females than in males when chromium was added to white sugar ($P < 0.001$).

Growth. The rates of growth of the rats given the Torula yeast, sugar and lard diet were somewhat lower than in those given the standard diet of rye, skimmed milk and corn oil. At 120 days of age, for example, males of the five groups on the former diet weighed 23 to 52 g less (females, 13 to 35 g less) than did rats on the standard diet. At 180 days of age, these differences

TABLE 2

Fasting serum glucose levels in rats fed three sugars and chromium

Sugar	Male			Female		
	Age	Serum glucose		Age	Serum glucose	
	days	*mg/100 ml* [1]	*P* [2]	*days*	*mg/100 ml* [1]	*P* [2]
Series 1						
White	150	106.7 ± 4.47	—	—	—	—
White + Cr	151	97.0 ± 2.90	< 0.05	—	—	—
Brown	157	86.0 ± 1.69	< 0.001	157	98.2 ± 3.02	—
White	324	104.4 ± 3.48	—	318	117.8 ± 3.38	—
White + Cr	322	109.4 ± 3.69 [3]	ns [4]	324	83.1 ± 4.56	< 0.001
Brown	318	85.6 ± 3.76	< 0.001	322	85.4 ± 4.13 [5]	< 0.001
Series 2						
White	128	121.6 ± 3.81	—	128	132.4 ± 3.79	—
"Raw"	128	110.4 ± 4.85	< 0.05	128	118.7 ± 5.14	< 0.025

[1] Mean ± SEM. Each mean is that of 12 rats randomly selected from each group. Chromium was supplied as indicated at 5 ppm in drinking water.
[2] P is significance of difference of mean from that of white sugar group.
[3] Differs from day 151 value, $P < 0.01$.
[4] Not significant.
[5] Differs from day 157 value, $P \sim 0.01$.

were 28 to 70 g and 16 to 60 g, respectively.

There were few significant variations in the mean weights of the five groups (table 3). Rats of series 2 fed raw sugar were usually smaller than were animals in series 1. Growth ceased in all rats of series 1 between 150 and 180 days of age and in all but one group of series 2 from 120 to 180 days of age. Cessation of growth did not appear to be seasonal, as it occurred in series 1 during February and in series 2 during August and September.

DISCUSSION

Rats obtaining 44% calories from refined white sugar and 30% from commercial lard (shortening) have relatively elevated fasting serum cholesterol levels compared with rats fed either white sugar supplemented with 5 ppm chromium(III) in drinking water or brown sugar isocalorically. Furthermore, there appeared to be a direct relationship between the amount of chromium in the sugar and the mean serum cholesterol at any age when measurements were made. From the percentage of ash in each sugar, the degrees of refinement were: brown < raw < white; chromium concentrations followed in that order. Therefore, chromium appeared to have an effect of suppressing serum cholesterol levels in rats, preventing a progressive increase with age which occurred only when refined white sugar was fed.

Although the rat does not ordinarily have hypercholesteremia unless fed cholesterol and saturated fats, relative values may be meaningful within moderate ranges, especially when other influences are applied. Thus, on our standard diet of seed rye, dry skim milk and corn oil, older rats showed lower serum cholesterol levels with added chromium than without (2). No marked increase with age was observed after maturity, although young rats less than 130 days of age generally showed somewhat lower levels than older ones, whether chromium was fed or not. Females required more chromium than males for circulating cholesterol levels to be suppressed (2).

The Torula yeast, sucrose and lard diet used in these experiments is low in chromium (0.1 μg/g wet weight); in rats fed this diet, the effect of chromium on circulating cholesterol was quite obvious. This effect also appeared in rats fed brown

TABLE 3

Mean weights of rats fed diet of Torula yeast, lard and various sugars with added chromium

Age	Series 1			Series 2	
	White Wt [1]	White + Cr Wt	Brown Wt	White Wt	Raw Wt
days	*g*	*g*	*g*	*g*	*g*
Males					
30	53 ± 1.7	57 ± 4.0	65 ± 3.9 [2]	48 ± 1.9 [3]	46 ± 3.0 [3]
60	127 ± 2.2	139 ± 11.4	130 ± 7.1	130 ± 5.9	119 ± 5.9
90	226 ± 4.5 [4]	227 ± 15.2	209 ± 8.7	217 ± 10.7	203 ± 9.7
120	275 ± 3.2	282 ± 16.9	271 ± 10.5	291 ± 12.4 [4]	261 ± 8.1
150	331 ± 9.8	323 ± 18.2	325 ± 15.0	283 ± 7.1 [5]	285 ± 12.0
180	330 ± 13.0	337 ± 25.2	321 ± 14.0	295 ± 7.7 [5]	298 ± 13.0
360	424 ± 5.0	417 ± 24.8	404 ± 13.2	—	—
Females					
30	57 ± 4.3	50 ± 1.0	59 ± 3.6	44 ± 2.2 [3]	46 ± 0.6 [3]
60	109 ± 11.4	117 ± 3.2	126 ± 5.9	110 ± 6.3 [3]	115 ± 3.2
90	185 ± 6.3 [4]	172 ± 3.1	176 ± 4.5	165 ± 4.5	164 ± 5.9
120	212 ± 5.9	199 ± 3.2	207 ± 5.5	195 ± 7.1	190 ± 3.2 [6]
150	230 ± 4.5	220 ± 2.2	235 ± 5.9	196 ± 9.3	193 ± 5.9 [3]
180	235 ± 6.3	229 ± 3.9	230 ± 8.1	195 ± 9.2	191 ± 4.5 [3]
360	249 ± 6.9	245 ± 17.2	258 ± 7.4	—	—

[1] Mean ± SEM.
[2] Differs from white, $P < 0.005$.
[3] Differs from raw, $P < 0.005$.
[4] Differs from brown, $P < 0.05$.
[5] Differs from white, series 1, $P < 0.01$.
[6] Differs from brown, $P < 0.025$.

sugar and to a slight extent in those fed raw sugar; brown and raw sugar both contained more chromium than did white. We have not evaluated all of the trace and bulk elements in the ash of sugar cane juice, unrefined sugar or molasses. We have no evidence, however, that other trace elements which may be present play important roles in the conversion of sugar or lard to circulating cholesterol by the rat which is fed adequate calcium, magnesium, potassium, sodium, iodine, iron, selenium and cystine, as provided in the salt mixture used. Rats fed our standard diet and exposed to 5 ppm nickel or niobium or 10 ppm molybdenum, however, showed low serum cholesterol levels (2).

In man, approximately 0.5% of chromium(III) chloride taken orally is absorbed by the intestinal tract (9). Trivalent chromium olates in alkaline media, forming long-chain molecules (10). Presumably the complex of chromium occurring naturally in biological materials is more readily absorbed, as chromium is ubiquitous in such materials (11). The proportion of chromic acetate absorbed by the rat intestine is not known, but is probably larger than that absorbed by man. If only 0.5% of the dose were absorbed by the rat, 5 ppm would supply 0.025 μg/ml, a concentration roughly 10% that in brown sugar; and 1 ppm, only 0.005 μg/ml, a concentration about 17% that in refined white sugar.

Supplements of 1 ppm chromium were sufficient for suppression of serum cholesterol in male but not in female rats (2). For example, the mean fasting level (in milligrams/100 ml serum) in females 660 days old given 1 ppm chromium was 116.0 ± 6.0; in those 405 days old given 5 ppm, 72.1 ± 5.3 ($P < 0.001$); and in those 480 day old ones given 12 ppm, 61.6 ± 2.2 ($P < 0.005$). These changes suggest increasing effects with dose in female animals. As exemplified by fasting serum glucose levels, a state of glucose metabolism resembling mild or moderately severe diabetes mellitus occurred in chromium-deficient rats, which could be restored to normal by feeding chromium(III) acetate (3, 12). These experiments utilized the standard diet of rye, milk and corn oil (5). Furthermore, male rats appeared to require more chromium than females to suppress fasting serum glucose levels. For example, mean levels of mature older rats fed or not fed chromium supplements in water were as follows: (in milligrams/100 ml serum) 1) males, 0 ppm, 137.2 ± 6.8; 1 ppm, 106.5 ± 3.6; 5 ppm, 101.1 ± 5.5 (at 414 days of age); and 5 ppm, 83.1 ± 2.8 (at 721 days of age). 2) Females, 0 ppm, 138.5 ± 5.7; 1 ppm, 79.6 ± 8.2; 5 ppm, 89.9 ± 2.1; 12 ppm, 75.4 ± 3.9. The carbohydrates were rye starch and lactose. Also, fasting levels tended to decrease with age when chromium was fed and to remain elevated when it was not.

In the present experiments the sole carbohydrate source was sucrose, in three degrees of refinement. Fasting serum glucose levels of male rats apparently responded poorly to chromium supplementation, whereas those of female rats responded well. Furthermore, brown sugar was more effective in suppressing fasting levels than was chromium-supplemented white sugar, suggesting that an additional hypoglycemic factor may have been present in brown sugar. Analyses done on sera of mature rats fed 12 trace elements suggested that in both sexes, nickel, arsenic and molybdenum were relative hypoglycemic agents.[12] Data on molybdenum in sugars are unavailable. Arsenic was present in lump sugar (0.1 μg/g) and in several raw sugars (0.5 to 2.03 μg/g), but not in brown sugar, white sugar, corn sugar or affination syrup. By atomic absorption spectrophotometry, there was 0.07 μg/g nickel in white sugar, 0.35 μg/g in brown sugar, < 0.02 μg/g in a very refined white sugar and 0.53 μg/g in an English brown sugar. Semiquantitative estimations of charred sugars by emission spectrography indicated that the brown sugar contained more copper, manganese, aluminum, sodium, magnesium and calcium than did the white.

As tissue chromium deficiency was common in the American, but not the Oriental, African and Middle Eastern, human subjects analyzed (4, 11) and as the feeding of chromium-deficient refined white sugar to rats was associated with relative elevation of circulating cholesterol levels, the data here reported support the hypothesis

[12] Schroeder, H. A., unpublished data.

that abnormalities in human cholesterol metabolism may be the result of a high consumption of refined sugar (1), indicating partial chromium deficiency. These rats will be observed until aged to ascertain possible pathological arterial lesions and the effect of chromium on such lesions.

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Role of Coprophagy in Utilization of Triglycerides, Calcium, Magnesium and Phosphorus in the Rat [1]

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ABSTRACT Absorption and retention of calcium, magnesium and phosphorus and digestibility of triolein, tripalmitin and tristearin in conventional rats and in rats prevented from coprophagy were compared. In a 2-week balance study carried out in 180 weanling rats fed three types of triglycerides (triolein, tripalmitin and tristearin) at either 5 or 25% level and three levels of calcium and phosphorus, prevention of coprophagy resulted in a marked depression in growth and a significant reduction in apparent digestibilities of tripalmitin, tristearin, calcium, magnesium and phosphorus ($P < 0.01$), but did not influence utilization of triolein. Retention of the three minerals, as tested by analysis of the femur, in rats prevented from coprophagy was identical to that of conventional animals. The effect of coprophagy should be considered in digestibility studies.

Previous studies on nutritional interrelationship of fats and calcium in rats have been carried out in animals which had access to their feces. Since rats consume up to 50% of their feces (1), it is important in studies of the utilization of triglycerides and minerals to compare results obtained in rats prevented from with those in rats allowed coprophagy. Barnes and coworkers (2) showed that prevention of coprophagy in rats increased the requirement for vitamin K, essential fatty acids and a number of B vitamins.

EXPERIMENTAL

Male weanling rats (180 animals) of Holtzman strain were maintained singly in wire-bottom cages in a room that was temperature-controlled at 25 ± 1°. Following 1 week for adaptation, the animals were divided into two groups. Each group was subdivided into 18 groups of five. To one-half of the rats, anal cups were affixed to prevent coprophagy (1); the other half carried the tail cups sufficiently far from the anus that feces could be consumed directly on extrusion.

The composition of the basal diet is shown in table 1. Calcium as calcium carbonate and phosphorus as potassium monobasic phosphate and triglycerides were added at the expense of cerelose. The fat content of the diets was 5 or 25% triolein, tripalmitin or tristearin. With each level and type of fat, three levels of calcium (0.08, 0.50 and 2.06%) and three levels of phosphorus (0.15, 0.58 and 1.67%) were used. Seven grams of food were allocated to each rat daily, but distilled water was provided at all times. All the rats consumed their daily ration, except that some of the rats fed 25% triolein had some leftover which was deducted from that offered.

Fecal cups were emptied daily for 7 days. The pooled 7-day fecal collection of each rat was weighed separately and kept frozen until analysis.

After 2 weeks, the rats were decapitated; one femur was isolated and cleaned. The fat in the femur was removed by toluene extraction for 2 hours and dried to constant weight. The femur, diet and feces were ashed at 600° for 16 hours and dissolved in 10% hydrochloric acid. Total fat was determined in diet and feces by Wistreich [3] apparatus using toluene extraction. Diets, feces and femur were analyzed for calcium, magnesium and phosphorus. The alkaline earth metals were analyzed by atomic absorption spectrophotometry, and phospho-

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[3] Delmar Scientific Laboratories, Maywood, Ill.

TABLE 1

Composition of the basal diet

	% of diet
Casein	20.0
Vitamin mixture [1]	2.2
Calcium-free salt mixture [2]	4.0
Cellulose flour	5.0
Chromic oxide	0.2
Cerelose	68.6

[1] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2] Mann Research Laboratories, Inc., New York, N. Y.

rus, by a modified method of Fiske and Subbarow (3).

RESULTS

Prevention of coprophagy resulted in a marked reduction ($P < 0.01$) in apparent digestibility of calcium, magnesium, phosphorus, tripalmitin and tristearin at any of the three levels of calcium and phosphorus tested; triolein utilization, however, was not affected (table 2). The improvement in digestibility of minerals as the result of coprophagy did not influence their retention in the femur, total ash in the femur, or moisture-free, fat-free weight of femur (table 3). Coprophagy prevention caused a marked reduction (about 20%) in total body weight gain in week 2 in those rats which received tripalmitin and tristearin but not those fed triolein.

DISCUSSION

Growth decreased about 20% in rats prevented from coprophagy despite the fact that they carried equally heavy cups and had a food intake equal to that of rats allowed coprophagy. This growth depression was due to decreased fat digestion and hence fewer calories derived from the ingested food. Prevention of coprophagy did not affect growth of rats fed triolein, which was almost completely absorbed, but did lower weight gain in animals fed tripalmitin and tristearin; this could be taken as evidence for this conclusion. A depression of similar magnitude was reported by Barnes et al. (4), although they attributed it to lower food intake rather than to decreased efficiency of food utilization.

Barnes et al. (5) did not observe any change in digestibility of Primex [4] (a hydrogenated vegetable shortening) fed to rats

[4] Primex, Procter and Gamble Company, Cincinnati, Ohio.

TABLE 2

Coefficient of digestibility of triglycerides and minerals by rats with coprophagy prevented and coprophagy allowed

Nutrient	Coprophagy prevented	Coprophagy allowed	Difference	t
	%	%	%	
Triolein	95.41 ± 1.30 [1]	96.45 ± 0.82 [1]	1.04	1.24
Tripalmitin	31.96 ± 6.53	42.37 ± 5.96	10.41	6.54 *
Tristearin	26.96 ± 1.15	36.97 ± 1.58	10.28	8.27 *
Calcium	50.18 ± 6.31	61.64 ± 6.61	11.46	6.26 *
Magnesium	42.18 ± 3.88	51.13 ± 4.61	8.95	5.11 *
Phosphorus	71.27 ± 2.36	77.40 ± 2.11	6.13	5.74 *

[1] Mean ± SE (mean).
* Highly significant ($P < 0.01$).

TABLE 3

Total body weight gain and mineral content of femur of rats with coprophagy prevented and coprophagy allowed

	Coprophagy prevented	Coprophagy allowed	Difference	t
Wt gain (2 weeks), g	20.00 ± 2.75 [1]	25.11 ± 3.02	5.11	7.06 *
Femur (dry, fat-free), mg	133.50 ± 19.80	133.61 ± 22.90	0.11	0.85
Total femur ash, mg	54.90 ± 15.50	56.42 ± 17.10	1.71	1.66
Calcium, mg	18.68 ± 1.41	18.62 ± 1.47	−0.06	0.23
Magnesium, mg	0.59 ± 0.09	0.65 ± 0.12	0.06	0.25
Phosphorus, mg	8.90 ± 0.56	8.73 ± 0.62	−0.17	1.27

[1] Mean ± SE (mean).
* Highly significant ($P < 0.01$).

at 7 or 15% of the diet. Their negative result may be due to the fact that in their experiment Primex was well digested (91.1 to 95.6%). We found similar results with triolein. When poorly absorbed fats were fed, such as tripalmitin and tristearin, coprophagy improved digestibility of fat to a marked extent ($P < 0.01$). In the experiment of Barnes et al. (5), food consumption was ad libitum, whereas, all our animals had equal food intake, thus eliminating the effect of level of intake on utilization of nutrients.

Digestibility of the three minerals tested was also improved significantly ($P < 0.01$) when rats were allowed to practice coprophagy.

In rabbits, it was reported that coprophagy improved digestibility of fat from purified diets, but not when roughage was provided; the digestibility of ash was improved in both types of diets (6).

These results introduce another factor to be considered when comparing the results of various investigators.

In the light of these observations, it becomes important to review digestibility studies of fats and minerals which have been conducted previously in the rat, rabbit and possibly other species which are known to practice coprophagy, such as mice, guinea pigs, dogs, swine and poultry. Since retention was not affected by coprophagy, it does not appear necessary to prevent coprophagy when the objective of the study is to investigate retention rather than digestibility.

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Interrelationship of Triglycerides with Calcium, Magnesium and Phosphorus in the Rat [1]

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ABSTRACT Two-week balance studies were carried out in weanling rats to investigate the effects of nature and level of triglycerides on calcium, magnesium and phosphorus metabolism and to study the effect of calcium, phosphorus and calcium-to-phosphorus (Ca/P) ratio on digestibility of triglycerides. Supplementation of fat-free diet with 25% tripalmitin or tristearin depressed calcium and magnesium absorption and retention, but had no effect on phosphorus metabolism. Diets containing 5% triolein, tripalmitin or tristearin or 25% triolein did not differ from the fat-free diet. Ca/P ratio of 0.05 or 13.75 lowered digestibility of tripalmitin and tristearin, but not that of triolein. Supplementation of 25%-tristearin diet with either 5% triolein, 5% monoolein, or 2.5% monoolein plus 2.5% oleic acid had no effect on absorption of tristearin, calcium, magnesium or phosphorus. Increasing dietary palmitin from 10 to 20% lowered calcium and magnesium absorption; a further increase of intake to 30% was without additional effect. Substituting 5% triolein for 5% tripalmitin improved absorption of calcium and magnesium at 5%- and 15%-tripalmitin intake, but not at 25%. Phosphorus metabolism was not affected in either case.

Many investigators in the last 50 years have studied the influence of fat and fatty acids on calcium absorption and utilization in different species, in various age groups, and under varied environmental and dietary conditions. Not unexpectedly, conflicting reports occur in the literature. Table 1 shows that some authors reported improvement in absorption and utilization of calcium when a low amount of fat is added to the diet (1–3). Others (4–8) observed that the utilization of calcium is largely independent of the level and the nature of fatty acids. A third group of investigators (9–20) demonstrated that fats have an inhibitory effect on calcium absorption.

Although conflicting reports still appear in the literature on the effect of dietary fats on calcium absorption, various investigators seem to agree on the reverse effect, that of dietary calcium on fat absorption. Excess calcium in the diet depresses absorption of fat in rats (21–25), rabbits (26), chicks (27), lambs [3] (28), dairy heifers (29), steers,[4] infants (30) and adult humans (31–34). The effects of dietary level of phosphorus and calcium-to-phosphorus ratio, however, have not been emphasized.

The effect of magnesium on lipid metabolism has been investigated intensively. An inverse relationship between the levels of magnesium and cholesterol in the serum of man (35) and rat (36) has been observed. Parenteral administration of magnesium sulfate to patients with abnormal lipoprotein pattern in coronary thrombosis has been shown to improve this disorder (37). Cheng et al. (22) reported that either magnesium or calcium lowered the digestibility of high melting point triglycerides and various hydrogenated fats. The reverse effect, that of fat on magnesium metabolism, however, has not received any attention. Seelig (38) reviewed the literature up to 1964 and concluded that there was insufficient data to define the effect of fat on the metabolism of this metal.

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TABLE 1

Effect of dietary fats on calcium absorption and retention

Reference	Diet	Species	Observation
McDoughall 1938 (1)	11% lard or olive oil + rachitogenic diet	rat	prevented rickets
Knudson and Floody 1940 (2)	5% cottonseed oil + rachitogenic diet	rat	better calcification
	10 to 20% cottonseed oil + rachitogenic diet		less effective
Jones 1940 (3)	10% lard + rachitogenic diet	rat	increase bone ash
Jenkins and Phillips 1960 (4)	3 to 20% fat	pup	no effect on Ca requirement
Steggerda and Mitchell 1951 (5)	1 to 32% fat	man	no effect
Nordin 1961 (6)	supplementary fat	man	no effect
Haymon et al. 1964 (7)	supplementary fat	man	no effect
Mallon et al. 1930 (8)	various levels of fat	woman	no effect
Smith and Spector 1940 (9)	5% mineral oil	rat	impaired bone calcification
Bunkfeldt and Steenbock 1943 (10)	cottonseed oil + rachitogenic diet	rat	impaired bone calcification
French 1943 (11)	5 to 45.5% oleo oil	rat	% Ca retention decreased as the level of fat increased
Calverley and Kennedy 1949 (12)	5% coconut oil or cottonseed oil	rat	decreased % Ca retained
	5% peanut oil		no effect
Kane 1949 (13)	high fat diet	rat	increase fecal Ca when older than 6 months
Beadles et al. 1951 (14)	20% cocoa butter	rat	decreased % Ca retention
	20% lard		no effect
Swell et al. 1956 (15)	20% oleic acid	rat	50% loss of Ca
	20% palmitic acid		less effective
Givens 1917 (16)	only poorly absorbable fat	dog	decreased absorption of Ca
Pepper et al. 1955 (17)	5 to 10% animal fat	chick	Ca requriement increased
White et al. 1958 (18)	supplemental fat	sheep	increased Ca requirement
Tillman and Brethour 1958 (19)	7.5% corn oil	sheep	decreased Ca retention
Filer 1967 (20)	formula with low digestible fats	infant	decreased retention of Ca

Little work has been done on the effect of fat on phosphate absorption. Telfer (39) reported that dietary fat caused greater absorption of phosphate by rats and dogs and concluded that fats form calcium soaps and thus reduce the amount of calcium available to form calcium phosphate which is excreted. Steenbock and Bunkfeldt (40) observed that the addition of 20% cottonseed oil to a rachitogenic diet containing 0.57% calcium and 0.09% phosphorus improved phosphate absorption and retention by the rat, but lowered the percentage of bone ash. They attributed this beneficial effect to the enhanced uptake of phosphate by soft tissues as a result of increase in growth.

The present study was undertaken to investigate the influence of the level and the nature of triglycerides on calcium, magnesium and phosphorus metabolism and the effect of calcium, phosphorus and calcium-to-phosphorus ratio on digestibility of triglycerides.

EXPERIMENTAL

In all experiments, weanling male rats of the Holtzman strain, weighing 47 to 60 g, were ear-notched and allotted to separate cages. Distilled water was provided ad libitum, but only 7 g diet/rat per day in experiments 1 and 2 and 8 g in experiment 3 was allowed; thus, they consumed all the diet. The animals were maintained on the experimental diets for 1 week to adapt to the regimen and environment. They were then transferred to metabolism cages, and tail cups were affixed to pre-

vent coprophagy, because coprophagy influences digestion (41). Tail cups were emptied daily; the 7-day fecal collection for each rat was pooled, weighed and kept frozen until analysis.

Experiment 1 was designed to study the effect of 0, 5, or 25% triolein (TO), tripalmitin (TP) or tristearin (TS) on fecal output, urinary excretion, serum concentration and femur content of calcium, magnesium and phosphorus at three levels of calcium intake (0.08, 0.50 and 2.06%), three levels of phosphorus (0.15, 0.58 and 1.67%), and constant intake of magnesium (0.51%). Four groups of rats (total 210 animals) were used. The composition of the basal diet is given in table 2. Calcium was added as calcium carbonate and phosphorus as potassium monobasic phosphate. All the additions were made at the expense of glucose.

In experiment 2, 20 rats were divided into four groups; zero, 5% TO, 5% monoolein (MO), or 2.5% MO plus 2.5% oleic acid (OA), was added to diets containing 25% TS in an effort to facilitate formation of mixed micelles, thus increasing absorption of tristearin.

In experiment 3, 30 rats were divided into six groups. Three of the diets contained 10, 20, or 30% TP. In the other three diets, 5% TO replaced 5% TP, giving TO/TP ratios of 1:1, 1:3 and 1:5. The total fat intake of the control groups in each case was kept the same as in the groups receiving TO as part of their fat intake.

At the end of each experiment, the rats were lightly etherized, decapitated and bled. The blood of each group was pooled, centrifuged and kept frozen (after removal of serum) until analyzed. The right femur of each rat was isolated, cleaned, defatted and dried to a constant weight.

TABLE 2

Composition of the basal diet

	% of diet
Casein	20.0
Vitamin mixture [1]	2.2
Calcium-free salt mixture [2]	4.0
Cellulose flour	5.0
Chromic oxide	0.2
Glucose	68.6

[1] In grams per kilogram: vitamin A conc, 4.5; vitamin D conc, 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; riboflavin, 1.0; menadione, 2.25; para-aminobenzoic acid, 5.0; niacin, 4.5; pyridoxine·HCl, 1.0; calcium pantothenate, 3.0; biotin, 0.02; folic acid, 0.09; vtiamin B_{12}, 0.00135; and thiamine·HCl, 1.0.

[2] In percent: magnesium chloride, 47.5; potassium citrate, 20.7; potassium chloride, 19.7; sodium chloride, 8.2; potassium sulfate, 2.1; ferric citrate, 1.6; potassium iodide, 0.04; sodium fluoride, 0.04; magnesium sulfate, 0.02; and potassium alum, 0.008.

Aliquots of diet and feces, as well as the whole femur, were ashed at 600° for 16 hours and dissolved in 10% hydrochloric acid. Diets, feces, urine, serum and femur were analyzed for calcium and magnesium by atomic absorption spectrophotometry and for phosphorus by a modified Fiske and Subbarow method (42). Total dietary and fecal fat was determined with Wistreich apparatus [5] using toluene extraction procedure.

RESULTS

Table 3 summarizes the effect of fats on calcium metabolism. The group fed the fat-

[5] Delmar Scientific Laboratories, Maywood, Ill.

TABLE 3

Effect of the level and the type of triglycerides on the metabolism of calcium

Treatment [1]	Ca [2] intake	Apparent Ca digestibility	Urinary Ca excretion	Femur Ca	Serum Ca
	mg/week	%	*mg/week*	*mg/whole femur*	*mg/100 ml*
Fat-free	560	62.05 ± 2.86 [a,3]	1.01 ± 0.16 [c,4]	17.50 ± 2.54 [bc,4]	10.4 ± 0.18
5% TO	560	51.43 ± 3.38 [ab]	0.84 ± 0.05 [bc]	19.08 ± 2.57 [ab]	9.6 ± 0.67
25% TO	560	58.40 ± 3.27 [ab]	1.14 ± 0.22 [c]	20.14 ± 3.36 [a]	10.5 ± 0.73
5% TP	560	48.43 ± 3.40 [ab]	0.08 ± 0.13 [bc]	19.16 ± 2.13 [ab]	9.6 ± 0.31
25% TP	560	25.43 ± 2.63 [c]	0.37 ± 0.08 [a]	16.25 ± 2.16 [c]	9.1 ± 0.53
5% TS	560	53.83 ± 3.48 [ab]	0.91 ± 0.07 [bc]	19.04 ± 3.71 [ab]	9.8 ± 0.42
25% TS	560	46.58 ± 2.83 [b]	0.65 ± 0.04 [ab]	16.39 ± 1.89 [c]	9.1 ± 0.36

[1] Rats were fed the basal diet shown in table 2 with triglycerides substituted at the expense of glucose. TO = triolein; TP = tripalmitin; and TS = tristearin. All values are means (30 rats) ± SE (mean).

[2] Average of six calcium levels.

[3] Means in the same column with different superscripts are significantly different ($P < 0.01$).

[4] Means in the same column with different superscripts are significantly different ($P < 0.05$).

free diet excreted the least fecal calcium, although not significantly different ($P < 0.05$) from the group receiving 5% TO. Absorption of calcium in animals fed 25% TP or 25% TS was significantly lower than in other groups ($P < 0.01$). Urinary excretion of calcium paralleled its absorption, decreasing with increasing levels of TP and TS; there was no significant difference among other groups. The serum level of calcium was constant regardless of the type or level of triglyceride fed. The calcium content of femur in the groups receiving 25% TO, 5% TO, 5% TP or 5% TS was higher ($P < 0.01$) than in the group fed the fat-free diet; this, in turn, was greater than those fed 25% TP or 25% TS.

Magnesium absorption was significantly correlated with fat absorption ($r = 0.434$, $P < 0.01$). The groups receiving either no fat or 25% TO excreted the least fecal magnesium (table 4). The three triglycerides tested, when administered at the 5% level, did not differ significantly from each other in their effect on magnesium absorption, but produced higher absorption of magnesium than 25% TP or TS. Urinary magnesium excretion paralleled its absorption; the lower the fecal excretion of magnesium, the higher its urinary elimination. Serum magnesium was highest in the group fed the fat-free diet, followed by the group receiving either 5 or 25% TO. Rats fed TP or TS had lowest serum magnesium concentrations ($P < 0.05$). Magnesium content of the femur was highest in groups fed at either 5 or 25% TO, and lowest in the group fed 25% TP.

It is of interest that no differences were observed in the phosphorus concentrations in feces, urine, serum or femur as a result of variation in the types and dietary levels of the triglycerides tested (table 5).

Fecal fat was highly correlated with weight of the feces ($r = 0.954$, $P < 0.001$). The percentage absorption of TO was higher at 25% than at 5% level. Unlike TO,

TABLE 4

Effect of the level and the type of triglycerides on the metabolism of magnesium

Treatment [1]	Mg intake	Apparent Mg digestibility	Urinary Mg excretion	Femur Mg	Serum Mg
	mg/week	%	*mg/week*	*mg/whole femur*	*mg/100 ml*
Fat-free	250	55.18 ± 4.50 [a,2]	23.87 ± 5.86 [ab]	0.535 ± 0.042 [c]	4.53 ± 0.60 [a]
5% TO	250	43.46 ± 4.94 [b]	19.29 ± 5.98 [b]	0.615 ± 0.052 [ab]	3.89 ± 0.23 [b]
25% TO	250	55.39 ± 6.26 [a]	36.12 ± 12.90 [a]	0.673 ± 0.049 [a]	3.87 ± 0.61 [b]
5% TP	250	42.42 ± 5.98 [b]	12.04 ± 7.24 [bc]	0.503 ± 0.088 [c]	3.13 ± 0.10 [c]
25% TP	250	30.74 ± 1.66 [c]	11.89 ± 0.27 [c]	0.410 ± 0.038 [c]	3.04 ± 0.10 [c]
5% TS	250	40.70 ± 8.22 [b]	21.91 ± 9.77 [b]	0.560 ± 0.058 [bc]	3.65 ± 0.54 [c]
25% TS	250	31.06 ± 4.17 [c]	11.91 ± 7.10 [bc]	0.545 ± 0.056 [c]	3.61 ± 0.30 [bc]

[1] Rats were fed the basal diet shown in table 2 with triglycerides substituted at the expense of glucose. TO = triolein; TP = tripalmitin; and TS = tristearin.
[2] Mean for 35 rats ± SE (mean). Means in the same column with the same superscripts are not significantly different ($P < 0.05$).

TABLE 5

Effect of the level and the type of triglycerides on the metabolism of phosphorus

Treatment [1]	P intake [2]	Apparent P digestibility	Urinary P excretion	Femur P	Serum P
	mg/week	%	*mg/week*	*mg/whole femur*	*mg/100 ml*
Fat-free	393.6	73.54 ± 4.85 [3]	110.21 ± 72.50	8.89 ± 1.44	9.0 ± 0.55
5% TO	393.6	72.44 ± 3.76	88.37 ± 58.60	8.08 ± 0.89	8.6 ± 0.42
25% TO	393.6	77.61 ± 3.79	91.32 ± 59.85	10.28 ± 1.55	8.2 ± 0.19
5% TP	393.6	69.67 ± 3.81	110.74 ± 64.09	8.77 ± 1.17	9.1 ± 0.25
25% TP	393.6	73.94 ± 4.55	98.48 ± 74.44	7.92 ± 0.91	8.2 ± 0.45
5% TS	393.6	74.56 ± 4.52	88.22 ± 58.17	8.35 ± 0.83	9.1 ± 0.36
25% TS	393.6	74.64 ± 4.21	111.33 ± 67.96	8.24 ± 0.78	8.2 ± 0.45

[1] Rats were fed the basal diet shown in table 2 with triglycerides substituted at the expense of glucose. TO = triolein; TP = tripalmitin; and TS = tristearin.
[2] Mean of six levels of phosphorus.
[3] Mean (35 rats) ± SE (mean).

TP and TS were poorly absorbed; they also caused more fecal excretion and less absorption when increased from 5 to 25% of the diet.

The level of calcium, phosphorus or calcium-to-phosphorus ratio had no effect on the amount of TO absorbed (fig. 1). Very low (0.05) or very high (13.75) calcium-to-phosphorus ratios, however, caused a significant reduction in percentage TP and TS absorbed.

Supplementation of the diets containing 25% TS with oleic acid derivatives had no significant effect on fecal and urinary excretion of calcium or on serum concentrations (table 6). Femur content of calcium, however, increased. Similarly, utilization of neither magnesium (table 7) nor phosphorus (table 8) was influenced by these supplements.

Increasing the level of TP from 10 to 20% decreased absorption and retention of calcium (table 9) and magnesium (table 10). A further increase in TP level to 30% had no additional effect. Substitution of 5% TP by TO caused an increase in percentage calcium and magnesium absorption. The level of dietary TP or its partial substitution by TO had no significant ef-

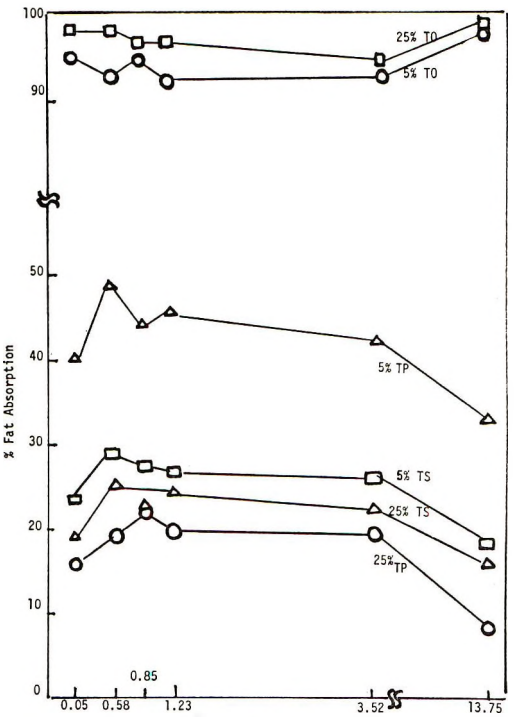

Fig. 1 Effect of calcium-to-phosphorus ratio (Ca/P) on the apparent coefficient of digestibility of triglycerides. TO = triolein; TP = tripalmitin; and TS = tristearin.

TABLE 6

Effect of supplementation of tristearin (TS) with triolein (TO), monoolein (MO) or oleic acid (OA) on calcium metabolism

Treatment [1]	Ca intake	Apparent Ca digestibility	Urinary Ca excretion	Femur Ca	Serum Ca
	mg/week	%	*mg/week*	*mg/whole femur*	*mg/100 ml*
25% TS	245	57.26 ± 4.62 [2]	0.36 ± 0.13	17.80 ± 0.75 [a,3]	9.1
25% TS + 5% TO	245	61.98 ± 1.68	0.42 ± 0.08	20.55 ± 0.60 [b]	10.0
25% TS + 5% MO	245	60.19 ± 2.93	0.31 ± 0.10	23.69 ± 0.27 [c]	9.4
25% TS + 2.5% MO + 2.5% OA	245	59.86 ± 2.67	0.44 ± 0.09	24.18 ± 0.38 [c]	9.1

[1] Rats were fed the basal diet shown in table 2 with substitutions at the expense of glucose.
[2] Mean (5 rats) ± SE (mean).
[3] Means with different superscripts are significantly different ($P < 0.01$).

TABLE 7

Effect of supplementation of tristearin (TS) with triolein (TO), monoolein (MO) or oleic acid (OA) on utilization of magnesium

Treatment [1]	Mg intake	Apparent Mg digestibility	Urinary Mg excretion	Femur Mg	Serum Mg
	mg/week	%	*mg/week*	*mg/whole femur*	*mg/100 ml*
25% TS	250	26.34 ± 5.13 [2]	0.62 ± 0.13	0.630 ± 0.010	3.30
25% TS + 5% TO	250	23.81 ± 5.35	0.39 ± 0.09	0.560 ± 0.040	2.88
25% TS + 5% MO	250	20.34 ± 5.81	0.51 ± 0.22	0.740 ± 0.030	2.38
25% TS + 2.5% MO + 2.5% OA	250	20.02 ± 2.79	0.60 ± 0.07	0.690 ± 0.010	2.92

[1] Rats were fed the basal diet shown in table 2 with substitutions at the expense of glucose.
[2] Mean (5 rats) ± SE (mean).

TABLE 8

Effect of supplementation of tristearin (TS) with triolein (TO), monoolein (MO) or oleic acid (OA) on phosphorus metabolism

Treatment [1]	P intake	Apparent P digestibility	Urinary P excretion	Femur P	Serum P
	mg/week	*%*	*mg/week*	*mg/whole femur*	*mg/100 ml*
25% TS	286.6	70.27 ± 1.36 [2]	25.96 ± 4.74	8.95 ± 0.26	8.2
25% TS + 5% TO	286.6	84.96 ± 0.84	30.20 ± 2.40	9.70 ± 0.20	8.7
25% TS + 5% MO	286.6	80.01 ± 1.48	32.10 ± 3.37	9.88 ± 0.92	8.2
25% TS + 2.5% MO + 2.5% OA	286.6	82.48 ± 1.35	23.60 ± 2.27	9.55 ± 0.20	8.3

[1] Rats were fed the basal diet shown in table 2 with substitutions at the expense of glucose.
[2] Mean ± SE.

TABLE 9

Effect of level of tripalmitin (TP) and its partial substitution with triolein (TO) on the metabolism of calcium

Treatment [1]	Ca intake	Apparent Ca digestibility	Urinary Ca excretion	Femur Ca	Serum Ca
	mg/week	*%*	*mg/week*	*mg/whole femur*	*mg/100 ml*
10% TP	364	47.52 ± 3.75 [ab,2]	0.53 ± 0.02 [a,3]	20.70 ± 0.55 [b,3]	9.6
5% TP + 5% TO	364	57.22 ± 5.24 [a]	1.28 ± 0.37 [b]	20.50 ± 0.50 [b]	10.0
20% TP	364	24.86 ± 4.94 [c]	0.53 ± 0.08 [a]	17.15 ± 0.67 [a]	9.0
15% TP + 5% TO	364	54.81 ± 2.76 [a]	0.48 ± 0.10 [a]	20.50 ± 0.00 [b]	9.8
30% TP	364	21.00 ± 1.78 [c]	0.19 ± 0.10 [a]	17.25 ± 0.45 [a]	8.4
25% TP + 5% TO	364	41.14 ± 6.47 [b]	0.53 ± 0.09 [a]	21.00 ± 1.04 [b]	10.0

[1] Rats fed basal diet shown in table 2 with triglycerides substituted at the expense of glucose. Mean (5 rats) ± SE (mean).
[2] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[3] Means in the same column with different superscripts are significantly different ($P < 0.01$).

TABLE 10

Effect of level of tripalmitin (TP) and its partial substitution with triolein (TO) on utilization of magnesium

Treatment [1]	Mg intake	Apparent Mg digestibility	Urinary Mg excretion	Femur Mg	Serum Mg
	mg/week	*%*	*mg/week*	*mg/whole femur*	*mg/100 ml*
10% TP	273	37.36 ± 5.59 [b,2]	0.05 ± 0.02 [b]	0.530 ± 0.016 [a]	2.62 [a]
5% TP + 5% TO	273	64.10 ± 0.79 [a]	0.23 ± 0.03 [a]	0.580 ± 0.013 [a]	2.62 [a]
20% TP	273	17.77 ± 5.14 [c]	0.05 ± 0.02 [b]	0.490 ± 0.021 [a]	2.30 [a]
15% TP + 5% TO	273	48.72 ± 2.79 [b]	0.06 ± 0.02 [b]	0.560 ± 0.019 [a]	2.62 [a]
30% TP	273	11.57 ± 5.19 [c]	0.05 ± 0.00 [b]	0.450 ± 0.025 [a]	2.12 [a]
25% TP + 5% TO	273	23.01 ± 7.51 [c]	0.05 ± 0.00 [b]	0.490 ± 0.018 [a]	2.55 [a]

[1] Rats fed basal diet shown in table 2 with triglycerides substituted at the expense of glucose. Mean (5 rats) ± SE (mean).
[2] Means in the same columns with the same superscripts are not significantly different ($P < 0.05$).

TABLE 11

Effect of level of tripalmitin (TP) and its partial substitution with triolein (TO) on the metabolism of phosphorus

Treatment [1]	P intake	Apparent P digestibility	Urinary P excretion	Femur P	Serum P
	mg/week	*%*	*mg/week*	*mg/whole femur*	*mg/100 ml*
10% TP	333.2	93.60 ± 0.94 [2]	123.30 ± 7.30	10.40 ± 0.37	8.5
5% TP + 5% TO	333.2	91.85 ± 1.38	115.63 ± 5.22	11.70 ± 0.30	9.0
20% TP	333.2	93.58 ± 2.62	148.50 ± 11.20	8.65 ± 0.48	8.6
15% TP + 5% TO	333.2	94.78 ± 0.36	111.00 ± 9.12	10.10 ± 0.38	9.6
30% TP	333.2	89.99 ± 1.12	137.40 ± 9.51	8.80 ± 0.32	9.0
25% TP + 5% TO	333.2	95.03 ± 1.35	112.10 ± 5.00	11.60 ± 0.22	9.3

[1] Rats fed basal diet shown in table 2 with triglycerides substituted at the expense of glucose.
[2] Mean (5 rats) ± SE (mean).

fect on phosphate absorption or retention (table 11).

DISCUSSION

When poorly absorbable fats constituted a large proportion of the diet, absorption of calcium and magnesium decreased considerably, regardless of the levels of calcium and phosphorus in the diet.

Studies from this laboratory [6] have shown by chemical and isotope balance studies, measurement of total body count and specific activities of ^{47}Ca in femur and tibia, that 20% TP or TS in the diet of rats receiving suboptimal levels of calcium (0.38%) and phosphorus (0.17%) had an adverse effect on the absorption and retention of calcium whereas TO or tributyrin were without effect.

In the present experiment the effects of triglycerides on the metabolism of calcium at three levels of intake, phosphate at three levels and magnesium at one level were studied. As in the previous study, a high dietary level of TP or TS caused a marked reduction in the percentage calcium absorption, regardless of which level of calcium and phosphate was fed; there was no significant difference when the 5% level of the three fats or the 25% level of TO were administered. It has been suggested that the adverse effect of fats on calcium absorption is due to increased excretion of calcium soaps (12, 14, 39). In our experiments high dietary levels of TP and TS caused a great increase in fecal volume. The alternative theory (15), that oleic acid formed large amounts of a calcium-oleic acid-phosphate complex which caused significant fecal losses of calcium, as compared with insignificant traces of complex when palmitic acid was substituted for oleic acid, cannot be substantiated from our data which showed that TO resulted in much better absorption of calcium than TP. Our results are in agreement with the findings of other investigators (16, 20, 43) who showed that only when fats are poorly absorbed do they depress calcium absorption. It has also been shown that calcium requirements of chicks (17) and sheep (18) increase in the presence of supplemental fat, suggesting a reduction in calcium retention.

Unlike the report of Kane (13), which stated that fat lowered absorption of calcium only in rats that were older than 6 months, we could show the effects in weanling rats. In our animals, however, coprophagy was prevented, eliminating recycling.

Even TO, which was thoroughly absorbed, did not enhance calcium absorption. Some investigators (1–3) have demonstrated that supplementation of diet with fats such as lard, olive oil or cottonseed oil increased bone calcification. They were, however, working with nonfat, low vitamin D, rachitogenic diets. The supplemental fat may have aided in the absorption of vitamin D and thus prevented rickets. It seems very unlikely that the effect had been directly on promoting calcium absorption. The theory (15) that fatty acids form absorbable soaps with calcium, and thus liberate phytin which otherwise would have formed an insoluble compound with calcium, was refuted by Jones (3) who did not find antirachitic action of lard salts but did with lard alone.

A number of factors have been shown to affect absorption of calcium and magnesium in a similar manner. Thus, absorption of both is lowered by dietary phosphate and phytate and raised by vitamin D, lactose, protein, antibiotics and growth hormone. We have demonstrated in this report that triglycerides also affect the absorption of these two alkaline earth metals similarly. Cheng et al. (22) reported that magnesium reduced digestibility of triglycerides which have melting point more than 50°, but the reverse effect, that of fats on magnesium absorption had not been studied previously.

Absorption and fecal excretion of magnesium were correlated with those of fats suggesting a quantitative relationship between the two. When the absorption of one was impaired, the absorption of the other was impaired to a similar extent. The effects of TP and TS were so marked that the concentration of magnesium was lowered in serum and femur. No homeostatic mechanism for regulation of serum magnesium is known (44), although the effects of parathyroid hormone and calcitonin on calcium homeostasis are well established.

[6] Unpublished observations.

This may explain the reduction in the concentration of serum magnesium but not that of calcium when large amounts of TP and TS were fed.

Neither phosphate absorption nor retention was affected by dietary triglycerides, regardless of the level of phosphorus or calcium in the diet. This finding contradicts other reports (39–40) which showed an increase in the amount of phosphate absorbed when diets contained fat. The discrepancy in results may be due to the prevention of coprophagy in our experiments.

It has been demonstrated in many species that excess calcium in the diet lowers absorption of fats. No mention has been made of the possible effect of the level of dietary phosphate. Our studies have demonstrated that when either calcium or phosphorus intake was high, while the other was low, absorption of the long-chain saturated triglycerides was decreased, but there was no effect on unsaturated triglycerides. The ingested fat may be hydrolyzed to free fatty acids, forming insoluble calcium soaps which are excreted, thus interfering with the utilization of both calcium and fat. When excess phosphorus is present in the alimentary tract, a calcium-fatty acid-phosphate complex may be formed, as suggested by Swell et al. (15), thus providing another excretory product of fat.

It is fairly well established that products of fat digestion, monoglycerides and fatty acids, combine with conjugated bile salts to produce micelles, the form of fat which is believed to be brought to the brush border of intestinal mucosa for absorption. Oleic derivatives, therefore, were added to diets containing 25% TS in an effort to facilitate formation of mixed micelles, thus increasing absorption of TS and possibly that of minerals. There was no improvement in the digestibility of TS. This is in accord with the results of other investigators (45) who did not observe any improvement in the absorption of stearic acid when supplemented with TO (67% stearic acid and 23% TO) and fed at 15% of the diet to rats. It was also demonstrated in experiment 1 that diets containing TO did not improve absorption of minerals, as compared with a fat-free diet. Putting these two findings together, it can be understood why the supplements did not influence utilization of minerals.

Experiment 3 confirmed the results of experiment 1, that high levels of a poorly absorbable fat (TP) depressed absorption and retention of calcium and magnesium with no significant effect on phosphate utilization. Furthermore, it demonstrated that when TO replaced part of TP intake, the depressive effect of the latter on calcium and magnesium utilization was diminished considerably.

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Control of Tyrosine-α-Ketoglutarate Transaminase Synthesis in Rat Liver: Studies of superinduction in force-fed rats [1]

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ABSTRACT Actinomycin D and cycloheximide caused an increase in tyrosine-α-ketoglutarate transaminase activity in rats depleted of dietary protein and force-fed with casein hydrolysate. In rats fed a high protein diet and then force-fed with glucose, the antibiotics did not have this effect. Insulin did not abolish the antibiotic-mediated increase of tyrosine-α-ketoglutarate transaminase activity in rats force-fed with casein hydrolysate. In the rats force-fed with glucose, tyrosine-α-ketoglutarate transaminase activity was decreased by two doses (100 μg/dose) of glucagon; and in these animals actinomycin D and cycloheximide caused a 10- and 5-fold increase, respectively, in tyrosine-α-ketoglutarate transaminase activity. In the animals force-fed with glucose, hydrocortisone acetate (5 mg) did not alter the activity of tyrosine-α-ketoglutarate transaminase; and in the glucocorticoid-treated animals, actinomycin D caused a two-fold increase in enzyme activity, whereas the effect of cycloheximide was negligible. The data were interpreted as an indication that increased glucagon release is necessary for the superinduction to occur, but that superinduction cannot be explained by assuming that the antibiotics stimulate glucagon release.

We have reported previously that the activity of tyrosine-α-ketoglutarate transaminase is increased by actinomycin D and cycloheximide (in rats prefed a high carbohydrate diet and force-fed with casein hydrolysate) more than the value obtained by force-feeding without the administration of the antibiotics (1). These increases did not occur in rats prefed a high protein diet and force-fed carbohydrate (1). Furthermore, 8-azaguanine (which inhibited the increase in glucose 6-phosphatase activity due to force-feeding casein hydrolysate) did not cause increased activity of tyrosine-α-ketoglutarate transaminase; nor did 8-azaguanine prevent the increased activity of this enzyme, when given in combination with actinomycin D or cycloheximide (1). These results did not rule out the possibility suggested by Garren and co-workers (2) that the induction of the transaminase is followed very shortly by the reestablishment of repression of tyrosine-α-ketoglutarate synthesis, although a satisfactory explanation could not be advanced to account for all the data (1).

Some of the data published by other workers (3–5) indicate that tyrosine-α-ketoglutarate transaminase can be induced by pancreatic hormones, even in the absence of exogenous or endogenous glucocorticoids; therefore, we have sought an explanation for the superinduction of tyrosine-α-ketoglutarate transaminase by actinomycin D and cycloheximide (1) which would take into consideration the dietary state and hormonal balance expected under the various conditions employed.

The following possibilities seemed appropriate to explain our results: 1) In the rats force-fed with casein hydrolysate, the release of glucagon should increase and the release of insulin decrease; the antibiotic-mediated superinduction could, therefore, be a result of increased glucagon or decreased insulin; if the latter, then the superinduction should be abolished by exogenous insulin. 2) In the rats force-fed with glucose, the hormone balance should be reversed and superinduction should be restored by exogenous glucagon. The following experiments were undertaken to distinguish between these possibilities.

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EXPERIMENTAL

Animals and treatments. Male rats of the Sprague-Dawley strain weighing 140 to 190 g were housed singly in screen-bottom cages and had ad libitum access to water. The animals were divided into two groups. One group was fed ad libitum for 4 days a diet consisting of 90% glucose, 5% corn oil, 4% Phillip-Hart salt mixture (6) and 1% vitamin mixture (7). These rats were then force-fed twice, at 10 AM and 10 PM, 4 ml 50% casein hydrolysate (enzymatically hydrolyzed) dissolved in distilled water per feeding. The animals force-fed casein hydrolysate were divided into three groups: Group 1 received no hormone treatment; group 2 was injected with 1 unit fast-acting insulin made from zinc insulin crystals;[2] and group 3 was given 2.5 units of the same insulin preparation. The hormone was administered intramuscularly in 0.10 ml water after the 10 AM force-feeding. Each group was further divided into three: 1) injected with two portions of physiological saline (1 ml each time), 2) injected with two doses of actinomycin D [3] (15 μg in 1 ml water/dose), and 3) injected with two doses of cycloheximide (25 μg in 1 ml water/dose). The injections were given intraperitoneally after each force-feeding.

The other group of animals was fed ad libitum for 5 days a diet consisting of 90% casein, 5% corn oil, 4% Phillips-Hart salt mixture (6) and 1% vitamins (7). Food was withdrawn 12 hours before force-feeding. The animals were force-fed three times, at 10 AM, 4 PM and 10 PM, with 4 ml 50% glucose dissolved in distilled water per feeding. The animals force-fed with glucose were divided into four groups: Group 1 received no hormone treatment; group 2 was injected with two doses (after the force-feedings at 10 AM and 10 PM) of 25 μg glucagon [4] dissolved in 0.05 M $NaHCO_3$; group 3 was injected with two doses of 100 μg glucagon (in the manner described above); and group 4 was injected with 5 mg hydrocortisone acetate [5] after the force-feeding at 10 AM. Each hormone preparation was injected intraperitoneally in a volume of 0.10 ml/dose. Each group was then subdivided into three groups: 1) injected with two portions of physiological saline (1 ml each time), 2) injected with two doses of actinomycin D (15 μg in 1 ml water/dose), and 3) injected with two doses of cycloheximide (25 μg in 1 ml water/dose). The injections were given intraperitoneally at 10 AM and 10 PM after force-feeding.

Procedure. Rats were killed by decapitation 24 hours after the first force-feeding, between 9:30 and 10:30 AM. Livers were quickly removed, rinsed, blotted, weighed and chilled over ice. Liver glycogen was determined by a nephalometric procedure (8). A 10%-liver homogenate was prepared with ice-cold 0.14 M KCl, pH 7.4, using a Potter-Elvehjem homogenizer. The crude homogenate was centrifuged at 0 to 4° for 30 minutes at 20,000 $\times g$, and the resultant clear homogenate was used for the determination of soluble protein (9) and the assay of tyrosine-α-ketoglutarate transaminase activity (10). The enzyme assay was performed at 25° using a multiple absorbance recorder.[6] Enzyme activity was expressed as units per 100 g body weight, and one unit of enzyme activity was defined as the amount of enzyme which can produce 1 μmole of product/minute under the conditions of the assay.

RESULTS AND DISCUSSION

The effects of actinomycin D, cycloheximide and insulin in rats force-fed casein hydrolysate are summarized in table 1. Relative liver size values were not affected appreciably by either the antibiotics or insulin. Liver glycogen values were depleted by force-feeding, and this effect was not reversed by further treatment. Soluble liver protein values were not affected by any of the treatments. Therefore, if the activity of tyrosine-α-ketoglutarate transaminase was expressed on a per-gram-liver basis or on a per-milligram-soluble protein basis, the percentage increases in enzyme activity due to various treatments would be approximately equal.

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[2] E. R. Squibb and Sons, Inc., New York, N. Y.

[3] Actinomycin D was a generous gift from the Merck, Sharp and Dohme Company, West Point, Pa.

[4] Crystalline glucagon was a generous gift from the Eli Lilly Company, Indianapolis, Ind.

[5] Suspension USP, Invenex, San Francisco, Calif.

[6] Model 2000, Gilford Instruments Laboratories, Inc., Oberlin, Ohio.

TABLE 1

Effect of insulin administration in rats force-fed casein hydrolysate

Treatment [1]	Relative liver size [2]	Liver glycogen	Soluble protein	Tyrosine transaminase
		mg/100 g body wt	*mg/100 g body wt*	*units/100 g body wt*
		Control		
PSP [3](8 [4]/9 [5])	3.65 ± 0.07 [6]	< 10	442 ± 9.0	2.17 ± 0.31
AcD (7/10)	3.48 ± 0.10	< 10	433 ± 16	20.2 ± 1.6
CHX (6/6)	3.50 ± 0.10	< 10	392 ± 8.0	25.3 ± 5.1
		Insulin-treated [7] (1 unit)		
PSP (2/2)	3.65	< 10	435	2.03
AcD (2/2)	3.77	< 10	444	12.2
CHX (2/2)	4.32	< 10	470	15.0
		Insulin-treated [7] (2.5 units)		
PSP (3/12)	3.90 ± 0.01	< 10	419 ± 15	3.35 ± 1.06
AcD (5/8)	3.58 ± 0.21	< 10	405 ± 35	16.0 ± 3.39
CHX (1/9)	3.65	< 10	438	35.9

[1] Rats were prefed a 90% glucose diet for 4 days. Following prefeeding, the rats were force-fed 4 ml 50% casein hydrolysate/force-feeding at 10 AM and 10 PM.
[2] (Liver wt × 100)/(body wt).
[3] PSP = physiological saline; AcD = actinomycin D; and CHX = cycloheximide. PSP (1 ml), AcD (15 μg in 1 ml of water), or CHX (25 μg in 1 ml water) was injected after each force-feeding.
[4] Number of animals surviving treatment.
[5] Number of animals treated.
[6] SE of mean.
[7] Insulin was administered intramuscularly in 0.10 ml water after the first force-feeding.

The activity of tyrosine-α-ketoglutarate transaminase was induced by actinomycin D and also by cycloheximide. Although insulin treatment may have caused some reduction in the antibiotic effects, the superinduction of the enzyme was not abolished. This indicates that the failure of the antibiotics to induce tyrosine-α-ketoglutarate transaminase in rats force-fed with glucose (1) is not a consequence of an increase in the release of insulin, although this may accentuate it. Higher doses of insulin caused 100% mortality within 2 hours after injection.

We then proceeded to test the assumption that glucagon is obligatory for the superinduction to occur and that the superinduction occurs due to an effect of glucagon and not by a stimulation of glucocorticoid release due to glucagon administration. Accordingly, rats force-fed with glucose were injected with actinomycin D or cycloheximide in the absence or presence of exogenous glucagon or hydrocortisone, as summarized in table 2.

As in previous treatments (table 1), relative liver size or soluble liver protein values were not appreciably altered by either the antibiotics or the hormones (table 2). Liver glycogen values were, however, reduced by the antibiotics, but not by glucagon; this suggests that the glycogenolytic effect of glucagon was masked because of the amount of glucose force-fed. In the control animals, a slight increase in tyrosine-α-ketoglutarate transaminase activity was noted due to the administration of antibiotics. These increases were not statistically significant. Similarly, no significant superinduction was observed at the lower dose of glucagon; however, superinduction was observable at the higher dose of glucagon. In addition, rats treated with 2 × 100 μg glucagon without antibiotics had a lower level of tyrosine-α-ketoglutarate transaminase activity which further accentuated the superinduction, particularly with actinomycin D. Hydrocortisone acetate did not produce superinduction when given in combination with cycloheximide, but did produce a slight superinduction with actinomycin D.

Glucagon (150 μg/100 g body weight) can cause a 400% increase in tyrosine-α-ketoglutarate transaminase in animals treated with anti-insulin serum, 24 hours

TABLE 2

Effect of glucagon and hydrocortisone administration in rats force-fed with glucose

Treatment [1]	Relative liver size [2]	Liver glycogen	Soluble protein	Tyrosine transaminase
		mg/100 g body wt	*mg/100 g body wt*	*units/100 g body wt*
		Control		
PSP [3] (10 [4]/10 [5])	3.97 ± 0.66 [6]	77 ± 12	437 ± 19	1.14 ± 0.12
AcD (3/4)	4.03 ± 0.27	65 ± 25	380 ± 21	1.30 ± 0.01
CHX (4/6)	4.45 ± 0.28	44 ± 19	456 ± 41	1.63 ± 0.26
		Glucagon [7] (2 × 25 μg)		
PSP (3/6)	4.26 ± 0.22	127 ± 14	400 ± 10	1.01 ± 0.10
AcD (2/3)	4.28	75	396	1.70
CHX (2/5)	4.18	90	400	1.11
		Glucagon [7] (2 × 100 μg)		
PSP (7/8)	4.05 ± 0.14	86 ± 5.0	392 ± 16	0.66 ± 0.05
AcD (5/6)	3.78 ± 0.18	77 ± 18	376 ± 29	6.56 ± 0.59
CHX (7/7)	4.24 ± 0.11	44 ± 3.0	421 ± 15	2.74 ± 0.62
		Hydrocortisone [8] (5 mg)		
PSP (4/4)	4.31 ± 0.20	146 ± 12	413 ± 14	1.30 ± 0.17
AcD (5/5)	4.38 ± 0.10	62 ± 18	431 ± 39	2.88 ± 0.39
CHX (5/7)	4.58 ± 0.33	44 ± 4.0	457 ± 22	1.33 ± 0.16

[1] Rats were prefed a 90%-casein diet for 5 days. Food was withdrawn 12 hours before force-feeding began. Rats were force-fed 4 ml 50% glucose/force-feeding at 10 AM, 4 PM and 10 PM.
[2] (Liver wt × 100)/(body wt).
[3] PSP = physiological saline; AcD = actinomycin D; and CHX = cycloheximide. PSP (1 ml), AcD (15 μg in 1 ml water), or CHX (25 μg in 1 ml water) was injected after force-feeding at 10 AM and 10 PM.
[4] Number of animals surviving treatment.
[5] Number of animals treated.
[6] SE of mean.
[7] Glucagon was dissolved in 0.05 M $NaHCO_3$ and administered in two doses (10 AM and 10 PM) intraperitoneally, in a volume of 0.10 ml/dose.
[8] Hydrocortisone acetate (5 mg in 0.10 ml) was administered intraperitoneally after the 10 AM force-feeding.

after treatment.[7] Furthermore, the circadian changes in tyrosine-α-ketoglutarate transaminase activity parallel the changes in blood glucose and not the changes in plasma corticosterone.[7] Also, the cycloheximide-induced increase in tyrosine-α-ketoglutarate transaminase (11) is mediated by the increased release of glucagon; this effect is counteracted by pentobarbital and pancreatectomy.[8] It appears, therefore, that the superinduction reported earlier (1) requires glucagon. This is consistent with the occurrence of the superinduction in rats force-fed casein hydrolysate, when glucagon release is expected to be increased, and the absence of the superinduction in rats force-fed with glucose, when glucagon release is expected to be decreased.

Exogenous glucagon alone (table 2), however, does not produce superinduction, but does so when administered with actinomycin D. This indicates that the role of the antibiotic in producing the superinduction cannot be explained by assuming that the antibiotic increases glucagon release. The data give further indication, therefore, that the induction of tyrosine-α-ketoglutarate transaminase synthesis is followed shortly by repression as suggested by Garren et al. (2) or by the production of a deactivator as suggested by Kenney (12). As to the role of glucagon in these processes, the data available do not allow a clear-cut interpretation.

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Influence and Degradation of Dietary Cellulose in Cecum of Rats [1]

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ABSTRACT Rats were fed diets containing three levels of cellulose to determine whether cellulose is an inert dietary diluent for monogastric animals. The addition of cellulose to a grain diet resulted in an increase in the quantities of volatile organic acids in the cecum. The organic acids determined were acetic, propionic and butyric acid which were probably the degradation products of cellulose. The increase of volatile organic acids in the cecum was primarily accounted for by butyric acid. The cecal contents of the rats fed diets containing higher levels of cellulose also increased. Cecectomized and intact rats were used to determine if the cecum is the primary site of cellulose degradation. The removal of cecum decreased the digestibility of cellulose; however, even cecectomized rats were able to digest substantial quantities of cellulose in a grain diet. The degradation products are postulated to be available to the rats. The three cecal volatile organic acids occurred in different proportions in the feces. This evidence suggests that the acids were absorbed or utilized differentially during passage from the cecum into the remaining large intestine.

Cellulose is a standard dietary diluent used in many nutritional studies. It is considered inert when fed to monogastric animals. There are, however, indications that cellulose is partially digested by these animals for they possess ceca or large intestines capable of bacterial fermentation which may be similar to that of the rumen. The observations that dietary cellulose is degraded by human beings and that the number of cellulolytic bacteria increased in the feces of the subjects support this suggestion.[2] In addition, large numbers of fungi capable of decomposing cellulose have been isolated from human stools (1). Further evidence that the microorganisms in the intestines of animals are directly responsible for the degradation of cellulose came from the addition of sulfathalidine in swine diets (2). The sulfa drug decreased the digestibility of cellulose from about 50 to 38%. In rats the intestinal microbes may play an additional role in the nutrition of the host animals by producing vitamins, especially the B-complex vitamins (3, 4). The addition of cellulose promoted the bacterial synthesis of riboflavin which was available to the rats (5).

Cellulose from various sources was digested by swine and rats (6, 7). The quantity of energy that might become available from cellulose digested by monogastric animals, however, has still to be discovered. In rats, as much as 50% of the cellulose was digested, and the products were absorbed and metabolized (8), based on the observation that about 50% of the radioactivity of the dietary cellulose appeared as radioactive carbon dioxide in the expired air. Other reports claimed that dietary cellulose did not contribute energy to the animals even though it was digested (7, 9–11). Perhaps a more correct interpretation would be that dietary cellulose increased fecal nitrogen or protein and that the extra fecal protein in terms of energy was in excess of the energy derived from cellulose digestion (10). Thus, in rats, the quantity of cellulose for optimal growth was 2% of the diet (12). When the concentration of dietary cellulose was 60%, most of the rats died. There is, therefore, no doubt that cellulose, especially at a high enough level, will exert effects more serious than producing a bulky and laxative effect, at least in rats.

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[2] Canham, J. E., R. S. Harding, C. F. Consolazio and N. F. Witt 1965 Gastrointestinal degradation of cellulose in the human. Federation Proc., 24: 314 (abstract).

Although the products of cellulose degradation in monogastric animals including human beings are not known, they are probably volatile organic acids. Under anaerobic conditions and depending on the pH and temperature, 80 to 90% of the carbon in the cellulose fermented in vitro is converted to acetate, butyrate, lactate and ethanol (13). Circumstantial evidence also indicates that, in rats as in ruminants, the products of fermentation occurring in the alimentary tracts are volatile organic acids. Concentrations of these acids in the cecum of rats were as high as those found in the rumina of cows or deer (14). Fermentation in the cecum of swine was claimed to be insignificant since removal of the cecum did not appreciably affect growth rates (15). It is doubtful that gross measurements such as growth rates are suitable indices of the significance of cecal fermentation. Furthermore, cecectomy decreased cellulose digestion by the pig in each of seven diets used in the experiments (15).

The present study was designed to secure information as to whether dietary cellulose will increase the quantities or change the proportions of the volatile organic acids in the cecum and feces of rats. Furthermore, balance studies were made to determine the digestibility of cellulose by cecectomized and intact rats.

METHODS

Eight adult male rats of the Sprague-Dawley strain, averaging from 314 to 322 g body weight, were used in each of three dietary groups. A basal grain diet,[3] nutritionally adequate in all respects, was fed to group 1. Groups 2 and 3 were fed diets in which part of the corn of the basal diet was replaced by cellulose [4] so that the final diets contained 5 and 10% added cellulose, respectively. The replacement of corn by even 10% cellulose was not expected to reduce the quality of the diet. Aside from carbohydrates, the major contribution of corn in the diet was protein; by calculation, the addition of 10% cellulose reduced the protein content of the basal diet from 22 to 21.3%. The three diets and water were fed to the rats ad libitum. Diet consumption was measured for each rat for seven days, 4 and 8 weeks after the initiation of the trial.

All rats were housed in separate suspended wire cages maintained at 27° in a room with 12 hours each of light and darkness.

After 9 weeks of feeding, feces from each rat were collected for 3 consecutive days. To stop bacterial fermentation the feces, once dropped by the rats, were collected in a 0.7 N H_2SO_4 solution. After the fecal collection period, the rats were not disturbed for 7 to 10 days except for feeding and caring; then all rats were decapitated at 9:00 A.M. Cecal contents were removed quickly and quantitatively and placed in chilled and tared containers. Immediately 0.7 N H_2SO_4 was added, of weight equal to the cecal contents, to stop further reactions. Acetic, propionic and butyric acids in the feces and cecal contents were determined by gas-liquid chromatography using standard curves obtained with known quantities of volatile acids.

In a separate study, two male and two female weanling rats (Sprague-Dawley strain) were cecectomized following the general surgical procedures recommended by Markowitz et al. (16). Four comparable rats, two from the same litter as those cecectomized, were sham-operated. All rats were fed the basal diet ad libitum for 40 days after the surgery. At the end of this time, the diet intake and feces produced during 12 days were recorded. The feces and representative samples of the basal diet were analyzed for cellulose by the acid-detergent method of Van Soest (17).

Data were analyzed statistically by analysis of variance (18).

RESULTS

No statistically significant differences were observed for the quantities of diets

[3] The percentage composition of the basal diet was: ground corn, 60.7; soybean meal (50% protein), 28.0; alfalfa meal (17% protein), 2.0; fish meal (12.5% protein), 2.5; dried whey (67% lactose), 2.5; limestone (38% Ca), 1.6; dicalcium phosphate (18.5% P, 23.5% Ca), 1.75; and iodized salt, 0.5. The following were also added: (in milligrams per kilogram feed) Mn, 121; Fe, 95; Cu, 7; Zn, 4; I_2, 4; Co, 2; choline chloride, 400; Ca pantothenate, 6; riboflavin, 3; niacin, 33; menadione, 2; DL-methionine, 500; penicillin, 2; streptomycin, 8; and arsanilic acid, 968; (in micrograms per kilogram feed) vitamin B_{12}, 7; and (in IU per kilogram feed) vitamin A, 8010; vitamin D_2, 750; and vitamin E, 5.

[4] Cellulose was Alpha Cel obtained from General Biochemicals Corporation, Chagrin Falls, Ohio.

consumed by the groups of rats. This was true whether the measurements were made after 4 or 8 weeks of feeding. The body weights of the rats in general reflected the quantity of feed consumed. Rats receiving the cellulose diets had body weights close to those fed the basal diet (table 1).

The wet weights of the cecal contents averaged 9.06, 9.64 and 11.13 g with standard deviations of 1.54, 1.28 and 1.90 g for groups 1, 2 and 3 (fed diets containing zero, 5 and 10% added cellulose), respectively. Those fed the 10% added cellulose had significantly greater cecal contents than those fed the basal diet ($P < 0.05$). Total quantities of volatile acids in the ceca increased from an average of 64.51 mg for group 1 to 71.76 and 78.64 mg for groups 2 and 3, respectively. The increases were contributed mainly by butyric acid (table 2). Total volatile acids in the feces averaged 64.44, 65.59 and 72.57 mg/rat per day for the three groups. The slight increase in fecal volatile acids of those fed the higher levels of cellulose was due to the three acids (table 2). Acetic, propionic and butyric acids must have been absorbed or otherwise intervened differentially dur-

TABLE 1

Average feed consumption and body weight of rats fed diets containing three levels of cellulose

Time on diet	Cellulose added to basal diet		
	0%	5%	10%
weeks	*food eaten, g/rat per day* [1]		
4	29.7 ± 4.7	27.9 ± 3.6	26.1 ± 3.7
8	26.2 ± 1.5	26.9 ± 5.6	27.8 ± 4.7
	body wt, g/rat		
0	322 ± 17	314 ± 10	321 ± 16
2	378 ± 16	379 ± 19	362 ± 25
4	428 ± 21	425 ± 26	408 ± 18
6	459 ± 12	450 ± 32	434 ± 24
8	498 ± 17	483 ± 36	469 ± 43

[1] Values are averages for eight rats ± SD; no significant difference in the amount of feed consumed or the body weights among the three groups of rats at any time of measurement ($P > 0.05$).

TABLE 2

Average quantities of volatile acids and their molar proportion in the cecum and feces of rats fed diets containing three levels of cellulose

Cellulose added to a basal diet	Acid		
	Acetic	Propionic	Butyric
%	*mg/rat, in cecum* [1]		
0	43.14 ± 10.8	8.62 ± 2.6	12.75 ± 4.5
5	41.52 ± 8.9	6.51 ± 2.0	23.73 ± 14.9
10	49.71 ± 14.9	6.51 ± 1.9	22.42 ± 5.8
	mg/rat per day, in feces [1]		
0	54.36 ± 10.0	4.71 ± 1.5	5.37 ± 1.7
5	55.58 ± 12.5	4.61 ± 1.5	5.40 ± 1.0
10	60.04 ± 16.1	5.28 ± 1.4	7.25 ± 2.3
	Cecal acid molar proportion, %		
0	73.3	11.9	14.8
5	65.9	8.4	25.7
10	70.7	7.5	21.7
	Fecal acid molar proportion, %		
0	87.9	6.2	5.9
5	88.2	5.9	5.8
10	86.7	6.2	7.1

[1] Values are averages for eight rats ± SD; the quantity and proportion of the individual acids in the cecum or in the feces are not significantly different among the three groups of rats ($P > 0.05$).

ing their passage in the large intestine to cause a difference in the molar proportion of acids between the cecal and fecal compartments (table 2). One explanation for the increased proportion of acetic acid in the rectum could have been a slower rate of absorption or utilization during its passage than for propionic and butyric acids. Another logical explanation is an increased rate of acetic acid production in the rectum, leading to the fecal increase.

Further evidence that dietary cellulose was not entirely inert was provided by the balance study which revealed that cellulose in the basal diet was digested. Digestibilities of cellulose by the four intact rats were 31.2, 42.9, 40.9 and 33.4%; by the cecectomized rats, 20.4, 14.6, 36.7 and 27.1%. The first two values in each of these were those of the male rats; the last two, the female rats.

DISCUSSION

The large intestine including the cecum receives ingesta which the upper sections of the gastrointestinal tract cannot digest. Therefore, any digestion which occurs in the large intestine indicates that this part of the intestine may play a unique role in the nutrition and the overall physiological economy of animals. Fermentation in the large intestine of indigestible ingesta from the upper sections of the gastrointestinal tracts is one way that "extra" energy may become available from cellulose which is often used to decrease the caloric density of experimental diets. The energy may become available when the products of cellulose degradation are absorbed. The work of Elsden et al. (14) would indicate that volatile acids were absorbed in the large intestine of rats, for the concentrations of these acids decreased in the rectum as compared with those in the cecum. This becomes even more important for animals that practice coprophagy. The recycling of feces permits the remaining unabsorbed products from degraded cellulose to be absorbed in the upper sections of the tract. The data from the present experiments are in support of this contention. Cellulose was partially digested by rats whether the cecum was present or not, although cecectomy decreased the digestion of cellulose. In addition, the higher the concentration of cellulose in the diet, the more are the cecal volatile acids.

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Amylase Synthesis in Pancreas of Rats Fed Soybean Flour

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ABSTRACT Fasted rats received one meal of soybean flour, either heated or unheated. After 3 hours, the pancreas of rats given the unheated flour contained considerably less amylase and produced in vitro slightly less of this enzyme than pancreas of rats given heated flour. Pancreas of nonfasted rats adapted to unheated soybean flour contained and produced less amylase than pancreas of rats adapted to heated soybean flour. A preparation of soybean trypsin inhibitor (SBTI) was injected into the duodenum of fasted rats following ligation of the pylorus. After 3 hours, less amylase was found in their pancreases than in those of controls. SBTI administered by stomach tube to pylorus-ligated rats did not decrease amylase content of pancreas. Thus, soybean trypsin inhibitors cause not only increased secretion but also decreased production of pancreatic amylase, and the stimulus is mediated through a humoral agent elicited in the duodenum.

Raw soybean flour (SBF) contains heat-labile trypsin inhibitors, to which other biological activities are attributed, such as growth inhibition and interference with cystine metabolism. Recently, attention was focused on their effect on the pancreas. Feeding unheated SBF causes pancreatic hypertrophy in rats and chickens (reviews, (1–3)). An effect closely related to hypertrophy, and perhaps its cause, is direct stimulation of the pancreas by the trypsin inhibitor. Thus, ingestion of unheated SBF by rats and chickens increases amylolytic, proteolytic and lipolytic activities of intestinal contents (4–6). Results obtained in our laboratory [1] (7) with rats given test meals of SBF indicate that less amylase and lipase are present in the pancreas and more in the intestinal contents, following one meal of unheated SBF than when the meal consisted of heated SBF.

This is a report on the in vitro synthesis of amylase in the pancreas of rats, adapted and nonadapted to either unheated or heated SBF, and on the site of the stimulus exerted by the trypsin inhibitor.

EXPERIMENTAL

Animals. Young male rats weighing 30 to 40 g of a local strain derived from Wistar rats were used throughout.

Soybean flour. Two types of SBF [2] were used: 1) unheated flour obtained from hexane-extracted unprocessed flour with excess solvent removed at room temperature; and 2) commercially processed (toasted) low fat flour.

Diets. Four experimental diets were used in which protein was provided by either casein, heated SBF or unheated SBF. They were prepared by incorporation of casein or the respective SBF into a protein-free basal diet,[3] previously described (8). The casein diets contained either 10 or 18% protein; the SBF diets, 10% protein.

Soybean trypsin inhibitor. A concentrate of soybean trypsin inhibitor (SBTI) was prepared according to Lyman and Wilcox (9). This SBTI preparation contained 3.2 trypsin-inhibitory units (TIU)/mg, as defined by Kunitz (10) and determined by Birk et al. (11). This corresponds to 0.15 mg trypsin inhibitor AA/mg plus

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[1] Konijn, A. M., Y. Birk and K. Guggenheim, unpublished data.

[2] Kindly supplied by Etz-Hazaith Ltd., Oil and Soap Factory, Petah Tiqva, Israel.

[3] The basal diet consisted of cornstarch, 91%; vegetable oil, 5%; and salt mixture USP XIV, 4%. It was supplemented with the following vitamins: (in milligrams per kilogram) thiamine, 2.0; riboflavin, 3.0; pyridoxine, 1.6; calcium pantothenate, 12.0; niacin, 30.0; and choline chloride, 1000. Each rat received 100 IU vitamin A and 4 IU vitamin D twice weekly.

0.33 mg Kunitz's soybean trypsin inhibitor/mg (3).

Test meals. Test meals consisted of a 25% (w/v) aqueous suspension of SBF, either heated or unheated. Two portions, each consisting of 5 ml/100 g body weight, were administered by stomach tube at a 20-minute interval.

Determination of amylase. The animals were killed by exsanguination. Their pancreases were rapidly removed, freed from extraneous tissue, weighed and homogenized with 20 mM phosphate buffer (pH 6.9) containing 5 mM $CaCl_2$ and 38 mM NaCl. The homogenized tissue was diluted with buffer as required for linear amylase activity. Amylase was estimated by a modification of the procedure of Bernfeld (12). Incubation was carried out for 10 minutes at 37°. The amount of amylase catalyzing the appearance of reducing groups equivalent to 1 mg maltose during an incubation period of 1 minute at 37° was defined as 1 unit of amylase. Amylase units were related to weight of pancreas and to pancreatic protein as determined by the method of Lowry et al. (13). Bovine albumin, fraction V [4] was used as standard.

Estimation of amylase synthesis. Amylase synthesis was studied with a modification of the procedure of Younathan and Frieden (14). The slices of pancreas were incubated in medium III of Krebs (15) at 37°. After 3 hours, they were homogenized in the medium and diluted with phosphate buffer; amylase was determined as described above. The difference between the amount of amylase present before and at the end of the incubation time was considered to be newly formed amylase.

RESULTS

In experiment 1 pancreatic amylase synthesis was studied in rats not adapted to SBF. They were fed a 10%-casein diet for 3 weeks. After a fast of 24 hours, one test meal of SBF was administered. Three hours after the second portion of the meal, the rats were killed. The amylase content of the pancreas was examined, and slices of pancreas were prepared for the study of amylase formation (table 1).

The pancreas of rats given one meal of unheated SBF contained significantly less amylase per gram tissue as well as per milligram protein, than that of rats treated with heated SBF. Tissue preparations synthesized less amylase per gram pancreas than slices from rats given heated SBF, the difference being almost significant. When amylase synthesis was expressed per milligram protein, no less amylase was formed by rats treated with unheated SBF. Apparently these rats produced relatively larger amounts of other proteins during the incubation period.

In experiment 2, amylase synthesis was studied in adapted rats, that is, in rats fed diets containing either heated or unheated SBF for 3 weeks; larger differences were observed (table 2). Pancreases of these rats were examined in the nonfasted state without being stimulated by a test meal. The difference in amylase content of the pancreas, per gram tissue and per milligram protein, was much larger than the difference between weights of pancreases. Furthermore, pancreases of rats given unheated SBF produced significantly less

[4] Sigma Chemical Company, St. Louis, Mo.

TABLE 1

Amylase synthesis by slices of pancreas of nonadapted rats 3 hours following one meal of either heated or unheated soybean flour

Soybean flour	Weight of pancreas	Amylase content of pancreas, 3 hr after test meal		Synthesis of amylase in vitro			
				per g pancreas		per mg protein	
	mg/100 g body wt	*units [1]/g pancreas*	*units/mg protein*	*units*	*% of initial content*	*units*	*% of initial content*
Heated	325 ± 40 [2]	3500 ± 310	15.7 ± 1.3	1190 ± 150	36 ± 13.5	4.2 ± 1.1	27 ± 3.5
Unheated	320 ± 32	1790 ± 80	8.6 ± 0.8	830 ± 100	47 ± 5.5	4.7 ± 0.5	58 ± 7.2

[1] One unit of amylase catalyzes the appearance of reducing groups equivalent to 1 mg maltose/1 minute incubation at 37°.

[2] All results are expressed as means ± SE of mean of 10 rats.

amylase per gram tissue and per milligram protein during the incubation period.

In experiments 3 and 4, the site of stimulation exerted by the active principle in SBF was investigated (table 3). Rats fed for 3 weeks an 18%-casein diet were fasted for 24 hours. They were anesthetized with ether; the abdomen was opened; and the pylorus was ligated. In experiment 3, they received two intraduodenal injections at a 20-minute interval; each injection contained 125 mg SBTI dissolved in 3 ml water/100 g body weight. Controls received SBTI that had been autoclaved at 9.1 kg pressure for 30 minutes. After 3 hours the rats were killed, and the amylase content of the pancreas was examined. Significantly less amylase was observed in pancreases of rats treated with unheated SBTI than in controls.

In experiment 4, rats were given the same amounts of SBTI by stomach tube. Pancreases examined after 3 hours showed no difference in amylase content between rats treated with the unheated or heated preparation. It appears, therefore, that the site of stimulation of pancreas by SBTI resulting in lower amylase content is the duodenum.

DISCUSSION

These results support the conclusion that unheated SBF or unheated SBTI depresses pancreatic amylase formation. Pancreases both of nonfasted adapted rats and of nonadapted rats fed a meal of SBF produced less amylase when the SBF offered was unheated than when heated. Moreover, injection of a SBTI preparation into the duodenum resulted in a diminished content of pancreatic amylase. Similarly, the rate of amylase synthesis in chick pancreas, as observed in vivo, was lower when unheated SBF had been fed than when autoclaved SBF had been fed (16). The total activity of pancreatic amylase, however, was higher when the chicks had been fed the unheated SBF, due to the larger size of the pancreas. The diminished amylase content of animals fed unheated SBF

TABLE 2

Amylase synthesis by slices of pancreas of adapted nonfasted rats

Soybean flour [1]	Weight of pancreas	Initial content of amylase		Synthesis of amylase in vitro			
				per g pancreas		per mg protein	
	mg/100 g body wt	*units [2]/g pancreas*	*units/mg protein*	*units*	*% of initial content*	*units*	*% of initial content*
Heated	489 ± 25 [3]	2630 ± 370	15.7 ± 2.2	830 ± 200	32 ± 5.1	4.7 ± 0.8	31 ± 4.0
Unheated	693 ± 34	380 ± 60	2.5 ± 0.4	120 ± 13	36 ± 5.8	1.0 ± 0.2	40 ± 9.2

[1] Rats had been adapted to diets differing only in type of soybean flour for 3 weeks.
[2] One unit of amylase catalyzes the appearance of reducing groups equivalent to 1 mg maltose/1 minute incubation at 37°.
[3] All results are expressed as means ± SE of mean of 10 rats.

TABLE 3

Amylase content of rat pancreas following pylorus ligation and administration of soybean trypsin inhibitor (SBTI)

Treatment of SBTI	Amylase [1]		
	units/g pancreas	*units/100 g body wt*	*units/mg protein*
Following injection of SBTI into duodenum			
Heated	10,150 ± 670 [2]	4940 ± 300	43.0 ± 2.3
Unheated	6300 ± 590	3140 ± 220	23.3 ± 2.5
Following administration of SBTI by stomach tube			
Heated	12,110 ± 1100	5700 ± 420	35.8 ± 3.6
Unheated	13,270 ± 1040	6550 ± 430	37.8 ± 2.6

[1] One unit of amylase catalyzes the appearance of reducing groups equivalent to 1 mg maltose/1 minute incubation at 37°.
[2] All results are expressed as averages ± SE of mean of seven rats.

is the result not only of depressed synthesis but also of increased secretion. The pancreases of chicks ingesting unheated SBF were depleted of zymogen granules to a greater extent than corresponding pancreases from chickens fed heated SBF (17). Furthermore, more amylase was observed in the intestine of rats (4, 7) and chickens (6) offered unheated SBF than when heated SBF had been fed. Lepkovsky et al. (6) explain this by the greater secretion by the pancreas, stabilization of amylase activity by raw soya bean, or both. The difference in amylase content of pancreas of rats maintained on either heated or unheated SBF was greater when the rats had been fasted.[5] The stimulus of food intake probably induces a greater amylase secretion in rats fed unheated SBF than in rats fed heated SBF. We interpret, therefore, the lower amylase content in pancreases of rats fed unheated SBF as a result of both depressed production and increased secretion.

The site of stimulus is probably the duodenum. The presence of SBTI in the duodenum appears to induce formation or secretion, or both, of a humoral factor stimulating the pancreas to increase secretion or to decrease production of amylase, or both. Such a factor has been postulated by Khayambashi and Lyman [6] on the basis of perfusion of rat pancreas with blood of a donor rat stimulated with SBTI. The humoral factor seems to resemble pancreozymin. Stimulation with pancreozymin likewise depletes the zymogen granule content of pancreatic cells and increases amylase output (18). Furthermore, pancreozymin, like SBTI, causes a significant pancreatic hypertrophy (19).

It has been assumed that the stimulation of the pancreas after ingestion of unheated SBF is a result of the presence in the intestine of less digestible and less hydrolyzed products of proteolysis (16). Our results with injection of SBTI into the duodenum of fasted rats lend no support to this assumption. The present observations are more nearly in conformity with the observations of Khayambashi and Lyman (20) who fed rats protein-free diets containing amino acids as source of nitrogen. Supplementation of this amino acid diet with SBTI induced pancreatic hypertrophy and increased pancreatic secretion.

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