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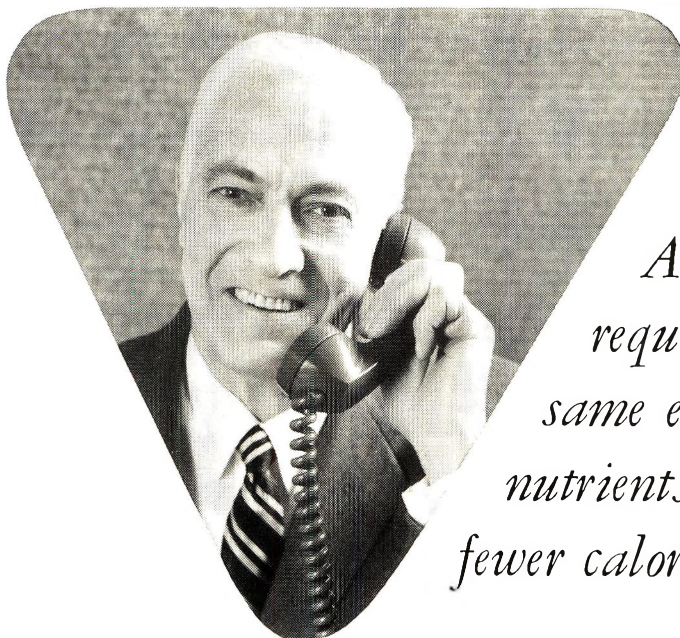

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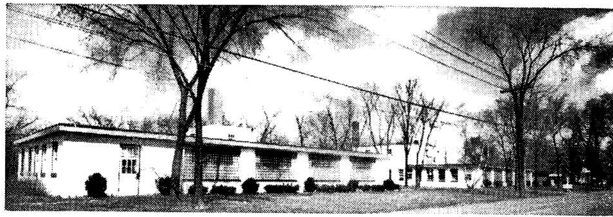

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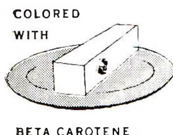

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kg	kilogram	km	kilometer
gm	gram	m	meter
mg	milligram	cm	centimeter
μg	microgram	mm	millimeter
mμg	millimicrogram	μ	micron
μμg	micromicrogram	mμ	millimicron
m^3	cubic meter	μμ	micromicron

Volume		*Area*	
cm^3	cubic centimeter	m^2	square meter
mm^3	cubic millimeter	cm^2	square centimeter
l	liter	mm^2	square millimeter
ml	milliliter		

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A	angstrom units	°	degree
%	per cent	ppm	parts per million

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John Raymond Murlin

1874 — 1960

John Raymond Murlin

1874 – 1960

Dr. John Raymond Murlin died March 17, 1960 in Spokane, Washington. He would have been 86 on April 30.

Doctor Murlin was a founder-member and past president of the American Institute of Nutrition. He edited the first 17 volumes of the Journal of Nutrition (1928–1939). In five decades he strove zealously and incessantly for the truths of nutritional science through teaching, writing and active laboratory investigation. One of his many outstanding accomplishments was the initiation and stout defense of the first research program to be established in the University of Rochester (1919). The academic climate at that time was at best unsympathetic toward such an uncomfortable innovation. Establishment of the University's Medical School in 1925 brought to Rochester a host of eager young investigators and shortly it became academically acceptable to do research. Dr. Murlin had broad interests and was versatile in skills and imagination. He encouraged his students in independent thinking and investigation and as a result they are found in physiology and biochemistry, as well as in nutritional science as such, in a wide variety of positions. One of his Ph.D.'s went on to win the Nobel Prize in chemistry.

His students and colleagues will miss his perennial curiosity concerning all sorts of vital phenomena and his great devotion to the ideas and ideals of science. He will always be remembered for his exuberant initiative and vigorous activity in his chosen field and for his gift of impelling others, by his own example, to become coworkers in the same broad field.

Biographical details may be found in J. of Nutrition, *31:* 1, 1946.

E. S. Nasset

Studies on the Interrelationships Between Dietary Magnesium, Quality and Quantity of Fat, Hypercholesterolemia and Lipidosis[1]

EARL E. HELLERSTEIN, MOTOOMI NAKAMURA,[2] D. MARK HEGSTED AND JOSEPH J. VITALE

Department of Pathology, Harvard Medical School and the Department of Nutrition, Harvard School of Public Health, Boston

Many studies have demonstrated that the quantity and quality of the dietary fat have a profound effect upon the serum cholesterol level in experimental animals and in human beings (Portman and Stare, '59). Generally, diets high in unsaturated fats tend to lower serum cholesterol levels whereas those which are saturated result in hypercholesterolemia. The mechanism by which fats influence serum cholesterol levels is obscure.

Recent studies from this laboratory have demonstrated that high dietary magnesium has an "anti-sudanophilic" effect in cholesterol-cholic acid-fed rats. Young weanling rats fed cholesterol and cholic acid developed lipid deposition in the left ventricular valves and aorta which could be partially prevented by increasing the level of dietary magnesium (Vitale et al., '57). This "anti-sudanophilic" effect of magnesium was not correlated with differences in serum cholesterol levels. All of these studies in which the "anti-sudanophilic" effect of magnesium was demonstrated were carried out with animals fed a hydrogenated cottonseed oil[3] as the sole source of fat.

The present experiment deals with the interrelationships between the amount of either a relatively saturated or an unsaturated dietary fat and varying levels of dietary magnesium upon the serum magnesium and cholesterol levels, vascular sudanophilia and other parameters of interest.

METHODS

Experiment 1. Young albino male rats, weighing 50 to 55 gm, obtained from the Charles River Breeding Laboratories, were fed a diet of the following constituents per hundred grams of diet: casein (purified), 10; glucose, 73.1; salt mixture, 5.0; celluflour,[4] 5.0; $CaCO_3$, 1.5; choline chloride, 0.3; a vitamin A, D and E mixture, 0.1; and fat, 5. The fats used were a hydrogenated cottonseed oil[5] (HCSO) or corn oil[6] (CO). When cholesterol and cholic acid as well as the higher level of fat (20%) were added to the diet, this was done at the expense of the glucose, gram per gram. The salt mixture was that of Jones and Foster ('42) with the calcium carbonate and magnesium sulfate removed. The vitamin A, D and E mixture contained 5 gm of halibut liver oil,[7] 1 gm of α-tocopheryl acetate and 34 gm of corn oil. The amounts of the water-soluble vitamins used in the diets have been listed elsewhere (Vitale et al., '57). Cholesterol was added at a level of 1.0% and cholic acid at 0.3%. The magnesium was added to the diet in the form of MgO to supply 24, 96 and 192 mg of magnesium per hundred gm of diet. The animals were fed ad libitum and housed in individual cages. At the end of 28 days, 6 animals from each group were killed and serum cholesterol levels determined by the method of

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[1] Supported in part by grants-in-aid from the National Institutes of Health, National Heart Institute (Grants no. H-3515 and H-3764), Public Health Service, Bethesda, Maryland; John A. Hartford Memorial Fund; Albert and Mary Lasker Foundation, New York; Nutrition Foundation, New York; The Age Center of New England; and the Fund for Research and Teaching, Harvard Department of Nutrition.

[2] Present address: Kyushu University Medical School, Fukuoka, Japan.

[3] Spry.

[4] Cellu Flour, Chicago Dietetic Supply House.

[5] See footnote 3.

[6] Mazola.

[7] Haliver oil.

Carpenter et al. ('57). Total serum protein was determined by the standard Kjeldahl method. Serum proteins and lipoproteins were determined by paper electrophoresis according to the method described by Moinat and Tuller ('57, '58). Serum magnesium was determined by the method of Orange and Rhein ('51).

Sections of kidney, heart and lesser curvature of the stomach from each animal were fixed in 10% formalin and then stained with hematoxylin and eosin. After the heart and aorta were opened so as to expose the left ventricular valves and aortic intima, they were fixed in formalin, stained with Sudan IV and graded as to the extent of sudanophilia of left ventricular valves and aorta (heart score). The left kidney of each animal was decapsulated and weighed. The extent of the microscopic calcium deposition in tubular lumens, mainly in the zona intermedia, the outer portion of the medulla, of the kidney was scored on the basis of zero to +4 (kidney calcium score) (Hellerstein et al., '57).

Experiment 2. At a later date, two groups of rats housed in individual cages were fed a diet containing 20% of corn oil but with the dietary cholesterol and cholic acid at considerably higher levels, 3 and 1%, respectively. Dietary magnesium was supplied at 24 or 192 mg/100 gm. The remainder of the diet and the methodology for serum cholesterol and "heart score" were the same as in experiment 1.

RESULTS

Experiment 1. Tables 1A and 1B illustrate the results of variations in the amount and kind of dietary fat and magnesium, with or without added cholesterol and cholic acid. The rate of weight gain appeared to be independent of the quality and quantity of fat fed and of the dietary level of magnesium. The addition of cholesterol and cholic acid to the diet resulted in decreased rates of weight gain. With the exception of the high-corn-oil group, the addition of cholesterol and cholic acid to the diet resulted in significantly lower serum magnesium levels when the dietary magnesium was 24 mg/100 gm. Increasing the level of dietary magnesium to 96 or 192 mg/100 gm resulted in a rise in the serum magnesium to approximately 2 mg/100 ml.

In the groups not consuming cholesterol-cholic acid containing diets (table 1A), increasing the level of dietary magnesium from 24 to 192 mg/100 gm resulted in decreased serum cholesterol levels in the groups fed 5 and 20% of HCSO. No such effect of magnesium was noted in either of the groups fed corn oil. The serum cholesterol levels of the groups fed 5% of HCSO were significantly lower than those of the groups fed 5% of CO, at each level of dietary magnesium. On the other hand, there was no difference in the serum cholesterol levels at any level of magnesium between the groups fed 20% of HCSO and 20% of CO.

When the animals were made hypercholesterolemic by the inclusion of cholesterol and cholic acid in the diet (table 1B), no consistent effects of magnesium upon the serum cholesterol levels were apparent. The serum cholesterol level in group 10 was significantly lower than the levels in groups 8 and 22, and the level in group 24 was significantly higher than in group 20 or 22. There are no consistent trends to suggest a logical interpretation. With the exception of group 10 and 24, the effects of HCSO and CO were those expected from previous work; i.e., increasing the level of the hydrogenated oil from 5 to 20% usually resulted in increased hypercholesterolemia; the reverse was true with CO, and animals fed CO had lower cholesterol values than those fed the HCSO.

The "anti-sudanophilic" effect of dietary magnesium was seen only in the groups fed the diets containing HCSO. At the 5% level of HCSO, the heart score of the animals in the group fell from 9 to 6 as the level of dietary magnesium was raised from 24 to 192 mg/100 gm. At the high level of HCSO, the heart score fell from 6 to 4 with increased dietary magnesium.

At the low level of dietary magnesium (24 mg%), rats fed the 20% HCSO diet had significantly higher serum cholesterol levels than rats fed the 5% HCSO diets. It should be emphasized, however, that the group fed 20% of HCSO had a significantly lower heart score ($P < 0.05$) than the 5% HCSO group. These observations held true for the high level of dietary magnes-

TABLE 1A

Effect of dietary fat and magnesium upon serum cholesterol, kidney profile and lipidosis of heart valves and aorta (no cholesterol or cholic acid added)

Group[1]	Dietary		Weight gain	Serum magnesium	Serum cholesterol	Kidney		Heart score
	Fat	Magnesium				Calcium score	KW/BW	
		mg/100 gm	*gm*	*mg/100 ml*	*mg/100 ml*		%	
1	5% HCSO[2]	24	78	1.08	105	1.2	0.48	0
3	5% HCSO	96	66	2.21	99	0	0.37	0
5	5% HCSO	192	71	2.56	83	0	0.38	0
7	20% HCSO	24	65	1.51	115	1.0	0.48	0
9	20% HCSO	96	78	1.89	113	0.2	0.38	0
11	20% HCSO	192	76	2.09	97	0	0.38	0
13	5% CO[3]	24	64	1.25	127	0.9	0.48	0
15	5% CO	96	63	2.03	138	0.2	0.40	0
17	5% CO	192	60	1.91	116	0.2	0.36	0
19	20% CO	24	76	0.96	115	1.0	0.50	0
21	20% CO	96	51	1.99	123	0	0.46	0
23	20% CO	192	76	1.95	102	0	0.38	0

[1] Six rats per group (28-day experiment).
[2] HCSO = Hydrogenated cottonseed oil.
[3] CO = corn oil.

TABLE 1B

Effect of dietary fat and magnesium upon serum cholesterol, kidney profile and lipidosis of heart valves and aorta (cholesterol and cholic acid added)

Group	Dietary		Weight gain	Serum magnesium	Serum cholesterol	Kidney		Heart score
	Fat	Magnesium				Calcium score	KW/BW	
		mg/100 gm	*gm*	*mg/100 ml*	*mg/100 ml*		%	
2	5% HCSO	24	56	0.88	724	0.8	0.54	9.0
4	5% HCSO	96	46	2.29	794	0.2	0.44	8.8
6	5% HCSO	192	51	2.22	821	0	0.41	6.2
8	20% HCSO	24	44	1.08	1086	0.8	0.50	6.0
10	20% HCSO	96	46	2.08	653	0	0.41	3.8
12	20% HCSO	192	53	1.91	1085	0	0.40	4.1
14	5% CO	24	42	0.98	397	0.8	0.58	3.6
16	5% CO	96	61	1.89	341	0.5	0.43	2.8
18	5% CO	192	53	2.15	385	0	0.40	5.3
20	20% CO	24	53	1.11	210	0.6	0.44	2.0
22	20% CO	96	45	1.99	270	0	0.42	2.3
24	20% CO	192	58	2.14	355	0	0.43	2.3

ium (192 mg/100 gm) groups, although the decrease in heart score was not statistically significant ($0.1 > P > 0.05$).

No "anti-sudanophilic" effect of magnesium was observed in the CO, cholesterol-cholic acid-fed groups. The overall average heart score for the low CO groups was 4. Increasing the level of CO to 20% resulted in a lower heart score of two regardless of the level of dietary magnesium.

The kidneys of the low dietary magnesium groups appeared speckled, pale, edematous and swollen. Increasing the level of dietary magnesium resulted in smaller kidneys which in the gross appeared normal. These gross signs of changes in kidney morphology appeared to be pronounced in the cholesterol-cholic acid-fed groups. Associated with this difference in gross appearance, there was a drop in the percentage of the body weight represented by the left kidney as the dietary magnesium was increased; i.e., group 1 versus group 3. The increased kidney weight-body weight ratio in the low-magnesium-fed animals did not appear to be a function of final weight or weight gain. In the group consuming 5% of HCSO and cholesterol and cholic acid, the weight gains of the low- and high-magnesium groups were similar but the kidney weight-body weight ratio was 0.54 at the low level of dietary magnesium and 0.41 at the high level of dietary magnesium. Thus, there was an absolute increase in kidney weight with low dietary magnesium. The absolute and relative drop in kidney weight with increased dietary magnesium was seen in all the groups with the exception of those animals consuming 20% of CO, cholesterol- and cholic acid-containing diets.

The microscopic appearance of the kidneys of the groups consuming high magnesium was always clearly differentiated from that of the low-magnesium-fed groups. This is expressed by the "calcium score." This score was approximately one in the animals fed a diet containing 24 mg/100 gm of magnesium and zero at the higher levels of magnesium. The histologic appearance of the kidneys and the deposition of fat in the aortic media have been described previously (Hellerstein et al., '57). A few animals among the various groups consuming low-magnesium diets (24 mg/100 gm) showed metastatic calcification of the gastric subserosal small arteries. A variable but small minority of rats fed the low magnesium diets have shown such calcification in our previous acute experiments.[8]

Tables 2A and 2B illustrate the effect of the type and level of fat and varying levels of magnesium on total serum protein and protein fractions as determined by paper electrophoresis. In general, in the HCSO-fed groups, the albumin levels decreased with the inclusion of cholesterol and cholic acid in the diet. Dietary cholesterol and cholic acid tended to increase the α-1 and β-globulin group (e.g., group 4 versus group 3).

Increasing the level of dietary magnesium resulted in decreased levels of both α- and β-lipoproteins in groups fed 20% of CO but not in the 5% CO, cholesterol-free groups. This effect of dietary magnesium was also noted in the 20 and the 5% HCSO group, although the absolute values were higher in the former.

The α- and β-lipoproteins were determined by paper electrophoresis in the cholesterol-cholic acid-fed rats but are not reported because of serious questions of methodology (see discussion section).

In experiment 2, increasing the level of dietary magnesium decreased the heart score from 5.2 to 2.9 ($P < 0.05$), at serum cholesterol levels of 440 and 385 mg/100 ml ($P > 0.05$) respectively. The weight gains of both groups of rats were identical.

Although experiments 1 and 2 were done at different times, it might be illegitimate to compare results of each.

The data in table 3 demonstrate, when compared with those in table 1B, that increasing the level of dietary cholesterol and cholic acid to 3 and 1%, respectively, in 20% CO diets, resulted in an increased serum cholesterol and heart score in the group fed 24 mg/100 gm magnesium diets but not in those animals fed the 192 mg/100 gm magnesium diets. The rats fed a 20% CO diet containing 1% of cholesterol and 0.3% of cholic acid and 24 mg/100 gm of magnesium had a serum cholesterol level of 210 and a heart score of 2.0 (table 2B). The animals receiving the dietary cholesterol and cholic acid at 3 and 1%,

[8] Unpublished observations.

TABLE 2A

Effect of dietary magnesium and quality and quantity of fat on serum protein and lipoprotein fractions (no cholesterol or cholic acid added)

Group	Dietary		Total protein	Albumin	Globulins				Serum lipoprotein[1]	
	Fat	Magnesium			α_1	α_2	β	γ	α	β
		mg/100 gm	%	%	%	%	%	%	*cm²*	*cm²*
1	5% HCSO	24	6.4	3.1	0.7	0.6	1.4	0.7	9.7	11.7
3	5% HCSO	96	6.1	2.9	0.9	0.5	1.1	0.7	8.5	10.3
5	5% HCSO	192	5.8	2.8	0.9	0.6	1.0	0.6	4.1	4.8
7	20% HCSO	24	6.3	2.8	1.1	0.6	1.2	0.7	15.9	11.8
9	20% HCSO	96	6.0	2.5	0.8	0.6	1.2	0.8	16.3	12.6
11	20% HCSO	192	5.9	2.6	0.8	0.6	1.2	0.8	7.7	6.3
13	5% CO	24	6.0	2.5	1.0	0.5	1.4	0.7	6.9	8.8
15	5% CO	96	6.3	2.9	0.9	0.6	1.3	0.7	10.0	10.6
17	5% CO	192	6.1	2.5	0.9	0.6	1.3	0.7	7.3	7.7
19	20% CO	24	5.8	2.3	0.9	0.6	1.4	0.7	8.1	8.5
21	20% CO	96	6.2	2.8	0.9	0.6	1.1	0.8	6.9	5.4
23	20% CO	192	5.7	2.3	0.9	0.5	1.0	0.8	4.0	4.8
Average			6.05	2.66	0.891	0.58	1.2	0.73		

[1] These values represent the total stainable lipid of the α- and β-lipoproteins determined by paper electrophoresis.

TABLE 2B

Effect of dietary magnesium and quality and quantity of fat on serum protein (cholesterol and cholic acid added)

Group	Dietary		Total protein	Albumin	Globulins			
	Fat	Magnesium			α_1	α_2	β	γ
		mg/100 gm	%	%	%	%	%	%
2	5% HCSO	24	6.7	2.8	1.1	0.6	1.4	0.8
4	5% HCSO	96	7.0	2.7	1.1	0.7	1.5	1.0
6	5% HCSO	192	6.6	2.6	1.1	0.7	1.4	0.9
8	20% HCSO	24	6.6	2.5	1.1	0.6	1.5	0.8
10	20% HCSO	96	6.3	2.4	1.1	0.6	1.4	0.8
12	20% HCSO	192	6.0	2.3	1.0	0.5	1.4	0.7
14	5% CO	24	6.7	2.6	1.2	0.6	1.7	0.7
16	5% CO	96	6.0	2.4	1.1	0.6	1.3	0.6
18	5% CO	192	6.3	2.5	0.9	0.6	1.5	0.8
20	20% CO	24	6.6	2.3	1.3	0.7	1.5	0.8
22	20% CO	96	6.0	2.3	1.0	0.6	1.3	0.7
24	20% CO	192	6.2	2.1	1.1	0.7	1.5	0.7
Average			6.42	2.46	1.09	0.62	1.45	0.78

TABLE 3

The effect of feeding 3% of cholesterol and 1% of cholic acid on serum cholesterol and heart score

Dietary		Weight gain	Serum cholesterol	Heart score
Magnesium	Fat			
mg/100 gm		*gm*	*mg/100 ml*	
24[1]	20% CO	46	440	5.2
192[1]	20% CO	46	385	2.9

[1] Fifteen rats per group.

respectively, had a significant increase in the serum cholesterol (440 mg/100 ml) and heart score (5.2). On the other hand, there was no effect of the increased dietary cholesterol and cholic acid either on serum cholesterol (387 mg/100 ml) or heart score (2.9) when the diet contained 192 mg/100 gm of magnesium.

DISCUSSION

High dietary magnesium may be "anti-sudanophilic" in the rat only where the serum cholesterol level is markedly elevated, somewhere in the range of 400 mg/100 ml or above, and where the heart score is high (above 3). In a recent study (Vitale et al., '57), in which thyroxine was fed to animals consuming a 10% protein and cholesterol-cholic acid containing diet, the low heart scores (approximately two) were not reduced further by the addition of dietary magnesium. Again the serum cholesterol level concentrations were below 400 mg/100 ml. This finding held in the present study in which the "anti-sudanophilic" effect of high dietary magnesium was observed only in those groups of animals in which the level of serum cholesterol was above 400 mg/100 ml. As in previous experiments (Vitale et al., '57; '59), the "anti-sudanophilic" effect of magnesium was not correlated with serum cholesterol levels.

The effect of CO on lowering serum cholesterol levels in humans and in animals is well known. However, not many studies have been conducted in which the effect of low and high levels of corn oil on the serum cholesterol levels have been studied. Recently, Hegsted et al. ('59) studied the interrelationships between the kind and amount of dietary fat and dietary cholesterol in experimental hypercholesterolemia. These investigators found that when the level of CO was 20% of the diet in which 0.45% of cholesterol and 0.45% of cholic acid had been added, there was, after 4 weeks, a significantly lower serum cholesterol level of 162 mg/100 ml compared with 310 mg/100 ml for the 5% CO-fed group. The difference between the high serum cholesterol levels seen in the present experiments (table 1B) and those recorded by Hegsted et al. ('59) is explained in part by the size of the animals used in their experiments. (In the present experiments 21-day-old male rats were used, whereas in those experiments recorded by Hegsted et al. ('59) young adult male rats, weighing 250 to 300 gm, were used.) These investigators further found that when the cholesterol was fed at a level of 1.35%, the cholic acid being held at 0.45%, there was no apparent effect upon serum cholesterol when the higher level of CO was fed (20 versus 5%).

In the present study, increasing the level of dietary cholesterol and cholic acid in animals fed 20% of CO resulted in no apparent increase in serum cholesterol of the high dietary magnesium group. There was, however, a significant rise in serum cholesterol of the rats fed low dietary magnesium. It is apparent that the level of cholesterol and cholic acid as well as the level of CO affects the serum cholesterol level. As Hegsted et al. ('59) have pointed out, the effect of most of these variables may be definable only in relation to many or all of the other variables.

In our previous studies (Vitale et al., '57; '59) and in this study, the "anti-sudanophilic" effect of high dietary magnesium in rats fed 20% of HCSO was not associated with a decrease in the hypercholesterolemia. In the present experiment, there was, at a constant level of magnesium (24 mg/100 gm), a higher lipidosis with 5% of HCSO than with 20% of HCSO, although the serum chol-

esterols were significantly higher with the 20% HCSO group.

Paper electrophoresis does not appear to be an adequate method for quantitating α- and β-lipoproteins in hyperlipemic, hypercholesterolemic serum. In our hands, the amount of lipid stained, quantitated by the use of a photoelectric densitometer, is not proportional to the amount of serum used to spot the paper. Secondly, reproducibility is extremely poor when hyperlipemic and hypercholesterolemic serum is used. Serum lipoproteins determined in animals fed diets devoid of cholesterol and cholic acid do not present these problems. Perhaps if one were to dilute the serum of cholesterol-cholic acid-fed animals with "mock serum" to a point where the concentrations of the lipoproteins were of the same order of magnitude found in the control animals, the values obtained might be more reliable and meaningful.

The α- and β-lipoproteins of the cholesterol-cholic acid-fed animals in the present experiment ranged from 2.5 to 7.5 and from 25 to 50 cm^2, respectively, regardless of the fat and level of magnesium. The values for these fractions are of the same order of magnitude previously reported (Vitale et al., '57).

Despite the unreliability of the method, the data would indicate that the inclusion of cholesterol and cholic acid in the diet resulted in decreased α- and increased β- lipoproteins. The results of the present and previous experiment (Vitale et al., '57), in which high dietary magnesium lowered the α- and β-lipoproteins in non-cholesterol-cholic acid-fed animals, suggest that one mechanism through which magnesium might exert an "anti-sudanophilic" effect could be related to lipoprotein metabolism. The results of an experiment dealing with the effects of dietary magnesium on serum lipoproteins determined by the ultracentrifuge method will be reported.

Many factors play an important role in the production of hypercholesterolemia; so, too, the "anti-sudanophilic" effects of thyroxine, magnesium, or other compounds may well be affected by the level and type of dietary fat consumed as well as by the quality and quantity of other nutrients.

The variation in vascular sudanophilia caused by various dietary constituents may rest not only on their influence upon serum lipids but also on their effects on the vascular wall. There are investigators, a minority, who believe that the primary lesion in arteriosclerosis or atherosclerosis lies within the media and is related to changes in the elastica or in the metabolism of mucopolysaccharides or collagen (Gillman et al., '55). Gillman ('59) has demonstrated changes in mucopolysaccharide metabolism in human atherosclerotic aortas and suggested the appearance of "abnormal" mucopolysaccharides. Shimamoto et al. ('59) demonstrated in the rabbit that infusion of large bacterial polysaccharides result in arteriosclerosis which could be prevented by the addition of dietary magnesium. In limited studies from this laboratory, we have observed changes in mucopolysaccharides from magnesium-deficient animals. Studies are now underway to test the effect of magnesium deficiency and excess upon mucopolysaccharides and collagen formation in normocholesterolemic and hypercholesterolemic animals.

SUMMARY

Young weanling rats were fed one of several diets which varied in the amount of magnesium (24, 96 or 192 mg/100 gm) and corn oil or a hydrogenated cottonseed oil (HCSO) (5 or 20%) added. The calcium content of the diet was 600 mg/100 gm. When used, cholesterol and cholic acid were added at one and 0.3%, respectively. The experimental period was 28 days. Increasing the level of HCSO from 5 to 20% usually resulted in increased hypercholesterolemia in rats fed cholesterol and cholic acid, whereas lowered cholesterol values were observed in rats fed a similar diet containing corn oil. The hypercholesterolemic response to cholesterol and cholic acid feeding was less marked in the animals fed corn oil. The "anti-sudanophilic" effect of high dietary magnesium was not related to decreased serum cholesterol concentrations. High dietary magnesium appeared to be "anti-sudanophilic" only when the serum cholesterol level was elevated and somewhere in the range of 400 mg/100 ml or above. Thus, no "anti-sudanophilic" effect of magnesium was noted in animals fed corn oil (5 or 20%) and 1% of cholesterol—0.3% of cholic acid. However, high magnesium

was "anti-sudanophilic" with 20% of corn oil when the serum cholesterol level was increased by feeding higher levels of cholesterol and cholic acid. The relationship of hypercholesterolemia and high dietary magnesium to lipidosis is discussed.

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The Effect of Dietary Magnesium and Thyroxine on Progression and Regression of Cardiovascular Lipid Deposition in the Rat[1]

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Increased dietary magnesium has been shown to result in decreased amounts of lipid deposited within the left ventricular valves and aorta of young male rats fed an "atherogenic diet" for approximately 25 days (Vitale et al., '57; Hellerstein et al., '57). To determine the effect(s) of feeding dietary magnesium over a longer time and whether the sudanophilia (lipid deposition) seen at 25 days was in fact a pre-atherosclerotic lesion, several groups of rats were fed atherogenic diets containing two levels of magnesium for a longer period. To determine whether sudanophilic deposits once established could be caused to regress, rats, after a priming period, were fed diets varying in magnesium and thyroxine. The present study demonstrates that heart valve-aorta sudanophilia, once established, can be reduced in the rat by feeding high dietary magnesium and/or thyroxine. In addition, in the region of aortic sudanophilia there developed grossly visible intimal plaques after 6 to 12 months.

METHODS

Male rats, 21 days old, obtained from the Charles River Breeding Laboratories, were fed a basic diet which consisted of the following in per cent: casein (purified), 10; hydrogenated vegetable oil,[3] 20; glucose, 56.9; celluflour,[4] 5; choline chloride, 0.3; salt mixture (Jones and Foster, '42), 5; cholesterol, 1; cholic acid, 0.3; and Ca carbonate, 1.5. The following vitamins were added in milligrams per kilogram of diet: thiamine·HCl, 4; riboflavin, 8; pyridoxine·HCl, 4; Ca pantothenate, 25; and niacin, 40. A mixture of vitamins A, D and E was prepared by adding 5 gm of halibut liver oil and 5 gm of α-tocopheryl acetate to 40 gm of corn oil[5] which was added to the diet at a level of 0.1%. Calcium carbonate and magnesium sulfate ($MgSO_4$) were removed from the salt mixture before using. Magnesium, in the form of magnesium oxide (MgO), was added to the basic diet at levels of 48 and 192 mg/100 gm. Thyroxine was added at levels of 1 and 2 mg/100 gm. The animals were fed and watered ad libitum and housed in groups of 8 in large cages.

At the end of 25 days, the animals from the various groups were decapitated. Serum cholesterol and magnesium were determined according to the methods of Carpenter et al. ('57) and Orange and Rhein ('51), respectively. Sections of kidney and heart from every animal, and selected tissues from many animals were fixed in 10% formalin for microscopic examination. After the heart and aorta had been opened to expose aortic and mitral valves and aortic intima, they were fixed in formalin, stained with Sudan IV and graded as to the extent of sudanophilia (heart score) (Vitale et al., '57). At this time, after the 25-day period, some of the animals fed a low-magnesium (48 mg/100 gm), thyroxine-free diet continued to be

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fed the diet without change. Others were assigned diets containing the same low level of magnesium but having added thyroxine (1 or 2 mg/100 gm). Still others were assigned diets containing higher levels of magnesium (192 mg/100 gm) with and without dietary thyroxine (1 or 2 mg/100 gm). Some animals continued to receive the original high-magnesium diet without change. Table 1 summarizes these dietary changes. After an additional 35 days, the animals in the various groups were killed and the above methods were again followed. Six animals maintained on a 48 mg/100 gm magnesium, cholesterol- and cholic acid-containing diet were killed at 6 months, two died at 9 1/2 months, and 4 were killed at 12 months.

TABLE 1

Dietary changes used in various experimental groups

Dietary (1st 25 days)		Dietary (26th–60th day)	
Magnesium	Thyroxine	Magnesium	Thyroxine
mg/100 gm	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
48	0	48	0
48	0	48	1
48	0	48	2
48	0	192	0
48	0	192	1
48	0	192	2
192	0	192	0

RESULTS

The animals fed the basic diet containing cholesterol and cholic acid and 48 mg/100 gm of magnesium without added thyroxine had a heart valve-aorta sudanophilia score of approximately 5 and a serum cholesterol level of 672 mg/100 ml after 25 days (table 2). A higher level of dietary magnesium, 192 mg/100 gm, resulted in a heart score of approximately three and a slightly higher serum cholesterol level of 851 mg/100 ml. The addition of thyroxine, 1 or 2 mg/100 gm, to either the low- or high-dietary-magnesium diets resulted in a heart score of approximately one and a serum cholesterol level ranging between 300 and 392 mg/100 ml.

TABLE 2

The effect of dietary magnesium and thyroxine on sudanophilia in rats fed cholesterol (1%) and cholic acid (0.3%) for 24 days

Dietary		Heart score[1]	Serum cholesterol
Magnesium	Thyroxine		
mg/100 gm	*mg/100 gm*		*mg/100 ml*
48[2]	0	4.8	672
48	1	1.2	291
48	2	1.1	327
192	0	3.0	851
192	1	1.3	392
192	2	1.0	362

[1] Sudanophilia of left ventricular valves and thoracic aorta.
[2] Six animals per group.

Table 3 illustrates the effect of adding thyroxine from the 25th day on, to either the low- or high-dietary-magnesium diets and the effect of adding more magnesium to the low-magnesium diets on total heart score and serum cholesterol levels. At the end of 25 days, the heart score in the 48

TABLE 3

Effect of change in dietary magnesium and thyroxine upon sudanophilia and serum cholesterol (end of 60 days)

Dietary		Total heart score		Serum cholesterol	
Magnesium	Thyroxine	25th day	60th day[1]	25th day	60th day
mg/100 gm	*mg/100 gm*			*mg/100 ml*	*mg/100 ml*
48	0 to 0	4.8	6.2	672	436
48	0 to 1	(4.8)[2]	2.0	(672)[2]	215
48	0 to 2	(4.8)[2]	1.5	(672)[2]	226
48 to 192	0 to 0	(4.8)[2]	4.8	(672)[2]	442
48 to 192[3]	0 to 1[3]	(4.8)[2]	1.6	(672)[2]	209
48 to 192	0 to 2	(4.8)[2]	2.0	(672)[2]	254

[1] Six rats per group.
[2] Assumed heart scores and serum cholesterol levels for animals not killed on the 25th day.
[3] These animals ate a diet containing 48 mg % of magnesium and no thyroxine for the first 25 days. On the 26th day the diet was changed to contain 192 mg % of magnesium and 1 mg % of thyroxine.

mg/100 gm thyroxine-free group was approximately 4.8, increasing to 6 at the end of the additional 36 days. However, when animals were changed to a high-magnesium diet on the 25th day, i.e., from 48 to 192 mg/100 gm, the heart score did not rise to 6.2 but remained at 4.8. At a high level of magnesium, 192 mg/100 gm, and with no added thyroxine, the sudanophilia score of three at 24 days actually improved with the score falling to 1.6 at the end of 60 days. These results bear no apparent relation to the serum cholesterol level at 60 days, for the averages were 436, 442 and 464 mg/100 ml, respectively. All groups which had thyroxine added to the diet starting on the 25th experimental day showed a decrease in heart score to one or two. The effect of thyroxine was apparently independent of the magnesium level or the amount of thyroxine fed. Serum cholesterol levels in the thyroxine-treated animals also decreased to approximately 200 to 250 mg/100 ml.

The aortic sudanophilia score contributed little to the total heart score (left ventricular valves and aorta sudanophilia) at 24 days but accounted for approximately one third of the total heart score at the end of one year (table 4). At the end of 25 days, the aortic sudanophilia score was 0.1 as compared with 5.6 at the end of one year, at which time gross intimal lesions were observed. The heart valve sudanophilia score seemed to have reached a peak at 200 days with a score of approximately 13 and with no further increase up to one year. Serum cholesterol levels, however, varied between 450 and 650 over the year period.

Only the rats fed the diet containing relatively low magnesium and no thyroxine showed renal calcium deposition, although only of a mild degree, at the end of 24 days. At 60 days, the degree of calcium deposition in the zona intermedia of the kidneys apparently had not increased. The animals maintained at the 48 mg/100 gm level of magnesium in the diet throughout the 60 days had a greater amount of renal calcium deposition and, in addition, cortical tubular dilatation than animals for which diets were changed at 24 days; i.e., increasing the level of dietary magnesium from 48 to 192 mg/100 gm and/or the addition of thyroxine to the diet for the last 36 days resulted in more normal appearing kidneys. The rats which had been fed the diet containing 192 mg/100 gm of magnesium for 24 or the entire 60 days showed virtually no renal calcium deposition or tubular dilatation.

As indicated in the methods section, of the rats fed the diet with 48 mg/100 gm of magnesium and cholesterol-cholic acid, 6 were killed at 6 months, two died at 9 1/2 months and 4 were killed at 12 months (table 4). One animal at 6 months and two at 12 months showed an intimal plaque in the arch of the aorta. Grossly, these plaques were gray-white, elevated above the level of the nearby intima and had a moderate amount of sudanophilic staining material at the periphery (fig. 1).

The plaque of two of the animals (one at 6 and one at 12 months) consisted almost entirely of cartilage. These contained numerous nests of typical cartilage-appearing cells; the stroma between the cartilage cells was metachromatic with toluidine blue (fig. 2). The intimal location of the plaques was shown unequivocally by the Verhoeff elastic stain (fig. 3). The aortic plaque of another animal, killed at 12

TABLE 4

Studies on the progression of vascular sudanophilia in rats from 24 to 365 days

Dietary magnesium	Days	Sudanophilia		Serum cholesterol
		Heart	Aorta	
mg/100 gm				*mg/100 ml*
48	24 (6)[1]	4.7	0.1	672
48	60 (6)	5.8	0.4	436
48	200 (6)	13.6	3.1	659
48	295 (2)[2]	13.5	4.0	—
48	365 (4)	12.0	5.6	485

[1] Numbers in parentheses indicate number of animals.
[2] These two rats died and were not sacrificed.

Fig. 1 Pearly gray plaque, in arch of aorta, with sudanophilic material (black) at periphery. Diet of rats: 48 mg% of magnesium for 6 months.

months, showed only rare nests of cartilage cells. The greatest portion of this plaque consisted of loose-to-fairly-cellular connective tissue (fig. 4). Towards the surface there were some cells, the nuclei of which were vesicular and somewhat blunt-ended. Although Heidenhain's aniline blue stain and Gomori's one-step trichrome stain showed a few of these cells to have the cytoplasmic color of muscle cells, we do not believe that any of them are necessarily smooth muscle cells. There was no calcium within the plaques, no fibrin adherent to the surface and no underlying medial calcification.

One animal in each of the 6-, 9 1/2- and 12-month groups showed enlarged kidneys with a marked internal hydronephrosis. The presence of the hydronephrosis had no apparent relationship to the development of aortic intimal plaques. Of the three animals showing this condition, only one had an aortic plaque. These kidneys contained numerous cortical cysts, some measuring up to 300 μ in diameter, often containing spurs of ruptured cyst wall extending into the lumen and surrounded by a mild fibrous reaction. Some cysts were filled with colloid. Calcium deposition was minimal in such kidneys; presumably, the calcium deposits that had initiated the process had been subsequently washed out. The remaining animals showed a small-to-moderate amount of calcium deposition in the

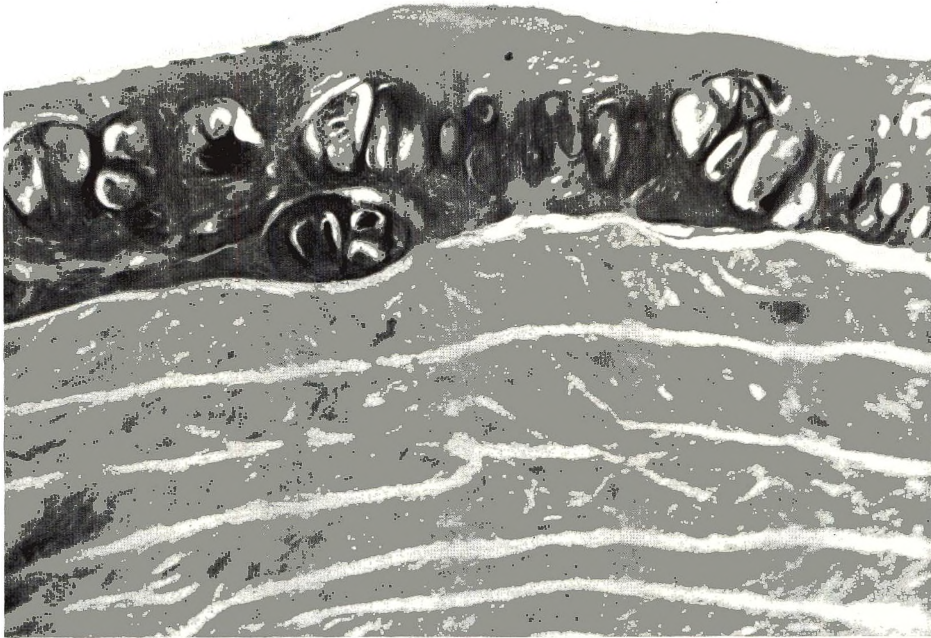

Fig. 2 Section through plaque in figure 1. Intimal plaque contains nests of apparent chondrocytes with intercellular material staining metachromatically. Toluidine blue. × 570.

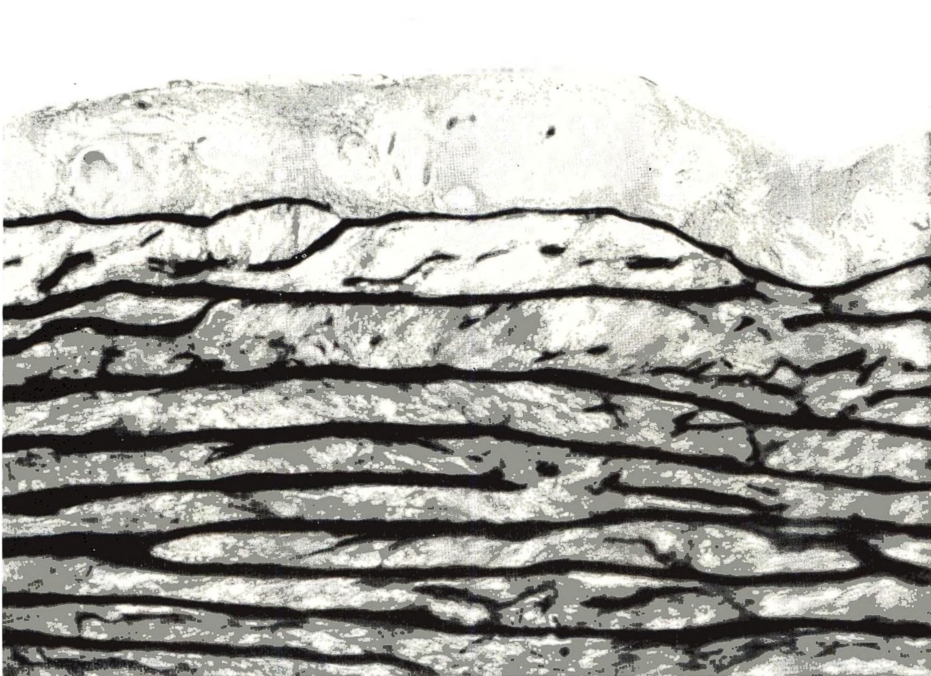

Fig. 3 Section through plaque in figure 1. Plaque is clearly intimal. Verhoeff van Gieson. × 570.

Fig. 4 Aortic plaque with loose stroma at base and spindle-shaped mesenchymal cells in upper portion. Rats fed diet for one year. Hematoxylin and eosin. × 570.

Fig. 5 Branch of renal artery. Calcium (black) replaces all of media in left half of picture. Despite 290 days of hypercholesterolemia-inducing diet, there was no intimal plaque above the calcified arterial media of these rats with internal hydronephrosis. Hematoxylin and eosin. × 600.

kidneys and only slight cortical tubular dilatation. Associated with the marked internal hydronephrosis was a remarkable picture in the numerous medium-sized and small pulmonic and systemic arteries. Calcium deposits impregnated the entire media in a patchy manner; longitudinal sections of arteries sometimes showed areas devoid of calcium alternating with areas where no medial nuclei could be seen, the media having been obliterated by calcium (fig. 5). Of interest was the fact

that despite all these areas of medial calcification in animals hypercholesterolemic for months, there was no intimal plaque and infrequent endothelial proliferation overlying such areas of calcification.

Most of the animals after 6 months showed calcium deposits in the interstitial tissue of the cartilage of the tracheobronchial tree. In some animals on long-term experiment the glomeruli contained clusters of foam cells. The lung capillaries and alveolar spaces contained xanthoma cells, neutral fat and birefringent crystals. This renal and pulmonary picture was similar to that previously described by Fillios et al. ('56) as was the mild cholesterol cirrhosis seen in two of the animals on experiment for one year.

DISCUSSION

The results of this study have confirmed those previously reported from this laboratory (Vitale et al., '57; '59; Hellerstein et al., '57) in which rats fed for 24 days diets containing cholesterol, cholic acid and high dietary magnesium had less heart-aorta sudanophilia than animals fed a similar diet except for lower magnesium.

Since the requirement of essential nutrients in general is decreased in adult animals, the possibility existed that the vascular sudanophilia seen at 25 days would not progress with continued ingestion of a diet containing 48 mg/100 gm of magnesium. The data presented, however, show that the heart score of animals fed this moderately low magnesium diet increased from 5 at 24 days to 6 at 60 days and to approximately 18 at the end of one year. No statement can be made about the effect of feeding rats high-dietary magnesium for a period of months, for none were studied past 60 days. Nevertheless, it is worthy of note that the heart score of the animals fed the high-magnesium diet decreased from three at 25 days to 1 1/2 at 60 days, this 60-day score being quite significantly lower than that of the animals fed the low dietary magnesium throughout that period. Furthermore, preliminary data on experiments extending well past two months and under conditions similar to those described in this presentation appear to indicate that the "anti-sudanophilic" effect on dietary magnesium is operative well past the one- and two-month stages.

Although the production of human and experimental atherosclerosis has been the subject of far more study than the question of its regression, the latter question has received some attention for the last 50 years.

Anitschkow ('28; '33) using rabbits, Bevans et al. ('51) using dogs and Horlick and Katz ('49) using chicks, were able to show regression in lipid-rich plaques of the aorta and/or other vessels upon removal of hypercholesterolemic agents in the experimental regime. Stukkeu ('11) noted no significant regression of atherosclerosis of rabbit aortas 4 to 6 months after the cessation of egg-yolk feeding. In rabbits fed cholesterol for three months and then a cholesterol-free diet for 6 months, McMillan et al. ('55) noted regression in neither visual grading nor cholesterol content of rabbit aortas despite a drop in serum cholesterol. Miller et al. ('59) found that the use of sitosterol in the diets of rabbits previously made hypercholesterolemic by dietary means caused a reduction in the hypercholesterolemia, yet did not cause regression of preformed aortic atheroma by the end of 24 weeks. All these results are probably compatible with those of Anitschkow ('28; '33), for the periods allowed for regression were not the prolonged one (1 1/2 to 3 years) used by him.

Regression experiments have not necessarily involved removal of the hypercholesterolemic agent; some have involved the addition of a substance; e.g., a hormone. Pick[6] found in chicks that estradiol injection induced regression of previously produced coronary lipid-laden plaques despite the continuation of the atherogenic stimulus, cholesterol-feeding. Cuthbertson et al. ('59) noted in rats an excellent correlation between sudan score and serum cholesterol concentrations. The extent of regression of such lesions, whether induced by a thyroactive drug or by a cholesterol-free diet, depended on the degree of depression in serum cholesterol concentrations.

[6] Pick, R. 1952 Estrogen-induced regression of coronary atherosclerosis in cholesterol-fed chicks. Federation Proc., *11*: 122 (abstract).

A mechanism by which dietary magnesium results in decreased amounts of sudanophilia is not clear. Certainly, the beneficial effects of high levels of dietary magnesium upon heart valve and aorta sudanophilia cannot be explained in terms of serum cholesterol levels for the results of this and other studies (Vitale et al., '57; '59) appear to demonstrate that high levels of magnesium result, if anything, in slightly increased levels of serum cholesterol. In contrast, the relatively well known "anti-sudanophilic" action of thyroxine may be explained, at least in part, by decreased levels of serum cholesterol associated with its ingestion. The results of these and previous studies strongly suggest that thyroxine, probably even more than magnesium—under the experimental conditions used—inhibits the development of and promotes the disappearance of lipid deposition within heart valves and aorta.

Hackel and Heymann ('59), using a different experimental model—rats with chronic renal disease produced by injection with rabbit anti-rat-kidney serum—also found arterial medial calcific lesions. They found no correlation between the presence of these calcific lesions and lipid deposits nor between the calcific lesions and the level of blood lipids.

The histologic appearance of the plaques of the aorta in animals on long-term experiment was in one respect quite unlike that seen in human arteriosclerotic plaques where intimal or, for that matter, medial cartilage is a rarity. Fillios et al. ('56) described the aortic plaques of their rats on long-term experiment as showing, among other things, proliferation of spindle-shaped mesenchymal cells. On occasion, cartilage-like cells were prominent in some of these plaques.[7] The question of the derivation of the cells in intimal plaques is beyond the scope of this paper except to indicate that both the fibroblasts and cartilage cells (such as seen in the rat plaque) are generally believed to be of mesenchymal origin.

It is quite clear that the sites of deposition of lipid in the cardiovascular system of the rat and human are quite different and that the cellular response of the rat aorta is different from that of man. We feel that these differences are, in all probability, those of species rather than of dietary difference. This is not to denigrate the use of the rat in the study of experimental arteriosclerosis, for much of potential value may be learned from using it, a convenient experimental model.

SUMMARY

Young male rats were fed an "atherogenic" diet (containing cholesterol and cholic acid) and two levels of dietary magnesium. The calcium content of the diet was 600 mg/100 gm. At sacrifice, rats fed low-dietary magnesium had a heart valve-aorta score (vascular lipidosis) of 4.8 at 24 days and a slightly higher heart score at the end of 60 days. If at the end of 24 days rats previously fed the low-magnesium diets were fed diets high in magnesium (192 mg/100 gm), there was no increase in the heart score at 60 days.

Animals fed high-magnesium diets had a heart score of only three after 24 days which was lowered to 1.5 at the end of 60 days. Thus, feeding high dietary magnesium appeared to result in some regression of vascular lipidosis as well as to exert an "anti-sudanophilic" effect. The addition of thyroxine to the diet caused a definite regression in vascular sudanophilia which was probably related in part to decreased serum cholesterol levels. There was a significant increase in the heart valve-aorta sudanophilia in the low-magnesium-fed rats after 6 to 12 months and several showed grossly visible aortic intimal plaques. Despite prolonged (6 to 12 months) hypercholesterolemia and the presence of extensive medial calcification of medium-sized arteries, such arteries exhibited no intimal plaque formation.

[7] Personal communication from S. B. Andrus, M.D.

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Physiological Aspects of Aging

III. THE INFLUENCE OF AGING ON CALCIUM METABOLISM IN RATS[1,2]

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Whereas the metabolism of calcium by growing and mature animals is clearly distinguishable when such criteria as calcium balance, bone density or radiocalcium uptake are applied, attempts to assess the changes which may be associated with time beyond the age of active accretion have yielded rather inconsistent results. Among other considerations, previous dietary history with respect to this element exerts a marked influence on the subsequent maintenance requirement, as demonstrated by Hegsted ('52) and Henry and Kon ('53). This influence may be a prime reason for the inconsistencies in the results obtained from different short-term studies in which the age groups under consideration were not all represented in each investigation.

The experiments of Henry and Kon ('53) demonstrated that aged rats fed a diet containing a liberal allowance of calcium went into negative balance when subjected to a low-calcium regimen, whereas essential equilibrium was attained if the animals had been previously adapted to a low calcium intake. The data of these investigators indicate, in addition, that no appreciable difference existed between rats 12 and 24 months old with respect to intestinal absorption of calcium. On the other hand, Hansard and Crowder ('57) found that the true digestibility of dietary calcium by rats declined from 41% at 48 to 72 weeks of age to 24% at 106 weeks, while the requirement for maintenance approximately doubled. Similar differences were observed by Hansard and co-workers ('54) between mature and aged cattle. In a study on dogs, Lloyd and McCay ('55) found no difference between animals two years and 9 to 12 years of age with respect to calcium retention.

In elderly people the amount of calcium required to maintain equilibrium has been variously reported to be greater than, or the same as, that of young adults. In men aged 69 to 88 years this requirement was found to be 18.5 mg per kg of body weight per day, and in women aged 48 to 80 years 16.7 mg per kg of body weight per day (Ackermann and Toro, '53, '54). This amount of calcium is considerably higher than the 8 to 12 mg required for attaining equilibrium in young adults, as reported by several investigators (Mitchell and Curzon, '39; Irving, '57). However, Bogdonoff et al. ('53) found that the amount of calcium needed for balance in aged males was in the range reported for young adults (850 mg per day) and that the magnitude of the negative balance with a low calcium intake was also similar. Ohlson et al. ('52), using data from an extensive study of calcium intake and retention by women aged 30 to 85 years, derived intake values for equilibrium which were similar for all age groups (averaging 875 mg per day) except those in the 8th decade, whose predicted requirement was lower. Malm ('58) also concluded on the basis of extended observations on 39 male subjects that no significant difference existed between men 20 to 50 years and 50 to 76 years old with regard to the efficiency of calcium absorption or degree of retention.

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[2] The experimental data in this paper are taken from a thesis submitted by the senior author to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the Ph.D. degree.

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The present study on rats was designed to investigate further the changes in calcium metabolism which may be associated with the aging process.

EXPERIMENTAL

Data on calcium absorption, turnover rate and endogenous excretion in 8 mature (10 to 12 months old) and 8 aged (22 to 32 months old) Sprague-Dawley rats were obtained by Ca^{45} and balance techniques. The rats were maintained during the pre-experimental period on a stock diet[4] containing 1.3% of calcium and 0.9% of phosphorus, with a supplement of 5,000 I.U. of vitamin A administered monthly. For one week prior to the administration of Ca^{45} and until the balance experiment was terminated, each rat was given triple-distilled water and was control-fed 5 gm daily of a diet having the following composition (%): vitamin-free casein,[5] 18.0; glucose,[6] 56.1; corn oil,[7] 6.0; cellulose,[8] 15.0; salts 446 (Spector, '48), 4.0; vitamin mixture in glucose (Draper et al., '58), 0.5; and choline chloride (25% in corn starch), 0.4. This diet contained 0.7% of calcium and 0.45% of phosphorus. At the beginning of the test period 5000 I.U. of vitamin A were administered. The animals ranged in body weight from 400 to 500 gm at the beginning of the preliminary period, without significant differences with respect to age, and were in negative energy balance during the experiment.

After a 12-hour fast a single tracer dose of high specific activity calcium chloride was administered by stomach tube in 0.5 ml solution. Urine and feces collections were made daily for 18 days using the apparatus described by Draper and Robbins ('56). Ashing of the samples and analysis for calcium were carried out according to the method of Norris and Lawrence ('53). Radioactivity in the sample was estimated by plating an aliquot of the ashed sample in dilute hydrochloric acid solution and counting in a Nuclear Model M-5 windowless gas flow counter attached to a Model 183B scaler.

The specific activities of the urinary and fecal calcium excreted each day were plotted on semi-log paper and straight lines were fitted by the method of least squares to the terminal exponential portion of the curve. During this period the two lines are parallel, that for the specific activity of fecal calcium being lower on the y axis owing to the dilution of Ca^{45} by unabsorbed dietary calcium (Blau et al., '54). The endogenous fecal Ca^{45} excreted during the absorptive period was estimated by extrapolating the line for fecal calcium specific activity toward zero, and the unabsorbed portion of the dose was calculated by subtracting this amount from the total fecal activity. The digestibility of the calcium in the experimental diet (a mixture of $CaHPO_4 \cdot 2H_2O$ and $CaCO_3$) was calculated from the ratio of the specific activity of fecal calcium to that of urinary calcium during the period of slow exponential decline (Hansard and Crowder, '57). This portion of the specific activity curve for fecal calcium was also used to estimate the biological half-life of plasma calcium.

RESULTS AND DISCUSSION

A typical plot of the specific activity of urinary and fecal calcium excreted daily for 18 days following an oral tracer dose of Ca^{45} is shown in figure 1. The specific activity of fecal calcium exhibited a maximum on the first or second day, then declined to a value equivalent to that of urinary calcium between the 4th and 6th days. The period of slow exponential decline usually was not reached until 9 to 11 days after administration of the dose, beyond which time the expected parallelism in the specific activity of urinary and fecal calcium was observed (fig. 1).

A summary of the data for average daily calcium intake, excretion, balance and endogenous excretion for the two groups of rats is given in table 1. Although the aged rats were found to excrete significantly more endogenous calcium (predominantly in the feces), both groups were nevertheless essentially in calcium balance during the 18-day period. The apparent explanation for this observation is found in the increased efficiency with which the aged rats

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[4] Purina Laboratory Chow, Ralston Purina Co., St. Louis.

[5] Labco, The Borden Co., New York.

[6] Cerelose, Corn Products Refining Co., New York.

[7] Mazola, Corn Products Refining Co., New York.

[8] Solka Floc, Brown Company.

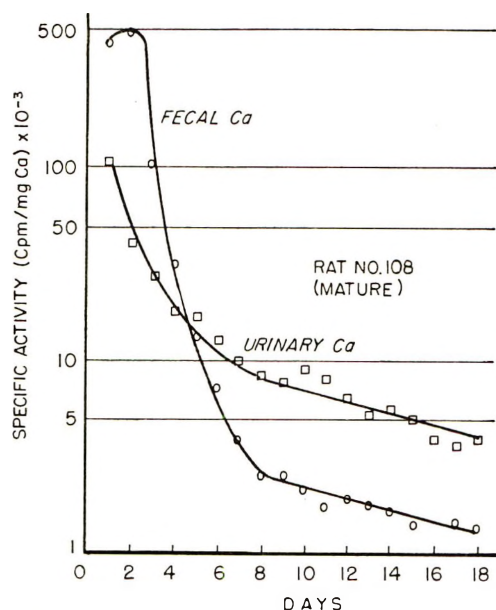

Fig. 1 Specific activity of urinary and fecal calcium following oral dose of Ca^{45}.

absorbed dietary calcium from the intestine (table 2). The results indicate that whereas the aged rats were subject to a greater loss of calcium from the body, they were able to compensate for this loss by an increased uptake from the dietary supply.

That at least part of the increased endogenous loss by the aged rats was due to poorer retention of calcium deposited in the skeleton is evident from a greater excretion of Ca^{45} during the terminal portion of the collection period. The total radioactivity excreted in the urine and feces from 12 to 18 days after the dose, expressed as a per cent of the counts administered, was significantly greater ($P < 0.05$) for the older animals.

The method used for estimating Ca^{45} absorption (table 2) is subject to errors which do not apply to the calculation of dietary calcium absorption. In contrast with the much shorter period generally believed necessary for gastrointestinal elimination, the apparent post-absorptive state with respect to the Ca^{45} dose was not attained in these experiments until the 9th to 11th day (fig. 1). In this connection Singer et al. ('52) have observed that after an intraperitoneal injection of Ca^{45} the fraction of the dose retained in the skeleton of rats declined rapidly for 10 days, then remained nearly constant for a further 80 days. They interpreted these results as indicative of the occurrence of skeletal calcium fractions with markedly different rates of turnover. These findings suggest

TABLE 1

Calcium intake, excretion and balance data for mature and aged rats

	Aged rats		Mature rats	
	Av.	S.D.	Av.	S.D.
	mg/day		*mg/day*	
Ca intake	34.0	2.1	34.2	1.3
Ca excreted				
urinary	0.55	0.35	0.9	0.3
fecal	32.9	3.2	33.1	1.2
total	33.4	3.4	34.1	1.1
Ca balance	+0.6	1.5	+0.2	1.0
Endogenous fecal Ca	15.4[1]	1.8	11.9	2.1
Total endogenous Ca	15.9	1.9	12.8	2.3

[1] Significantly greater than for mature group ($P < 0.01$).

TABLE 2

Estimated true absorption of dietary calcium and Ca^{45} dose by mature and aged rats (%)[1]

	Aged rats		Mature rats	
	Av.	S.D.	Av.	S.D.
Dietary calcium	48.8[2]	9.2	38.0	6.15
Ca^{45}	39.6	4.5	34.1	8.9

[1] See text for method of calculation.
[2] Significantly greater than for mature group ($P < 0.05$).

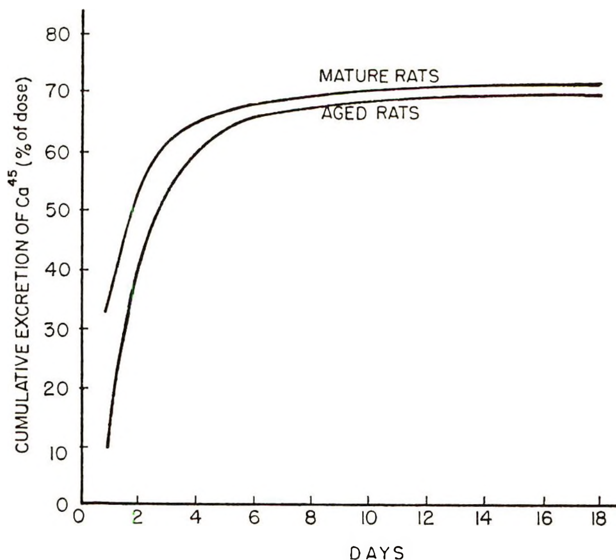

Fig. 2 Cumulative excretion of Ca^{45} in urine and feces following oral dose of Ca^{45}.

that during the first 9 to 11 days of the present experiments the plasma calcium (and hence fecal and urinary calcium) was being labeled by the relatively high specific activity calcium present in the labile fraction of the skeleton. Estimation of endogenous fecal Ca^{45} by extrapolation toward zero of a line fitted to the terminal portion of the curve for the specific activity of fecal calcium therefore underestimates the endogenous Ca^{45} excretion and gives a spuriously low value for Ca^{45} absorption. This error may be reflected in the lower values obtained in these studies for Ca^{45} absorption than for dietary calcium absorption, and in the failure to find a significant difference in Ca^{45} uptake between age groups.

Cumulative Ca^{45} excretion curves for the mature and aged groups are shown in figure 2. They demonstrate that about 70% of the administered dose was excreted during the 18-day experimental period. A significant difference ($P < 0.05$) in the fraction of the dose excreted on the first day (32.6% for the mature versus 9.7% for the aged rats) possibly indicates a slower rate of food passage in the old animals or the influence of some other factor such as rate of consumption of the diet offered after the dose. The older rats were observed to be generally more susceptible to psychic disturbances which were sometimes reflected in their eating habits.

The apparent biological half-life of plasma calcium, estimated from the rate of decline in specific activity of fecal calcium during the last 7 to 9 days of the experimental period, was found to be 12.4 ± 1.0 and 11.4 ± 1.0 days for the aged and mature rats, respectively ($P > 0.05$). This measurement represents the net effect of several factors upon the rate of decline in the specific activity of the plasma calcium pool, including the rate of intestinal absorption of Ca^{40} and of the endogenous excretion, as well as the rate at which plasma calcium is labeled by exchange with skeletal Ca^{45}. In view of the increased efficiency of dietary calcium absorption by the old animals and the overall calcium balance, it might be anticipated that the half-life of plasma calcium would be reduced in these rats. A probable explanation for the failure to observe a reduction lies in the continuous labeling of plasma calcium by exchange with Ca^{45} in the skeleton. Since the loss of skeletal Ca^{45} was found to be greater in the aged rats, the specific activity of plasma calcium would tend to be maintained at a higher level, thereby counteracting the isotope dilution resulting from the increased absorption of dietary calcium. These considerations signify that values derived by this

procedure for the half-life of plasma calcium fail to represent the true turnover rate, although they probably approximate it.

Whereas the results obtained in these experiments are in agreement with those of Hansard and Crowder ('57) for rats of similar ages with respect to the decreased retention by old animals, these authors found that the intestinal absorption of calcium also declined, thereby markedly increasing the maintenance requirement. In contrast with the calcium balance observed in aged rats receiving 34 mg of calcium daily in the present studies, these workers reported a maintenance requirement of 92 mg per day. The absolute figures for calcium absorption and endogenous loss are not comparable owing to the wide disparity (about 2.5 times) in Ca intake; however, the aged animals used in the two studies appear to have been in significantly different physiological states with respect to their ability to adapt to the increased endogenous loss. The present study indicates that the aged animal is capable of adapting to the increased physiological demand for calcium resulting from an accelerated skeletal catabolism by enhancing the uptake from the intestine, in a manner analagous to the increased absorption effected by the growing animal in response to the need for skeletal accretion.

SUMMARY

Rats representing two age groups (10 to 12 months and 22 to 32 months) were used in a study of the influence of aging on calcium metabolism. The endogenous excretion as well as intestinal absorption of calcium were observed to be significantly greater in the aged animals. The retention of calcium incorporated into the skeleton, measured by Ca^{45} labeling, was significantly poorer in the older group; however, both groups were found to be in overall calcium balance. These results indicate that the aged rats were subject to a greater loss of calcium from the body but were able to maintain calcium equilibrium by increasing the uptake of dietary calcium from the intestine.

ACKNOWLEDGMENT

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The Amino Acid Composition and Nutritive Value of Proteins

III. THE TOTAL PROTEIN AND THE NONESSENTIAL AMINO NITROGEN REQUIREMENT[1]

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The essential amino acid requirements of the growing rat for optimum growth have been redetermined in our laboratory (Rama Rao et al., '59). The total protein (N × 6.25) level in all these studies was maintained at 10% of the diet.

To assess the optimum total protein level when each of the essential amino acids is fed at the minimum required level, growth data and nitrogen retention were obtained at varying protein levels by supplementing the casein-amino acid diet with glycine or a mixture of nonessential amino acids. These studies are reported in this paper.

EXPERIMENTAL

Groups of male Sprague-Dawley rats (50- to 60-gm weight), individually housed in raised wire screen cages, were fed ad libitum the casein-amino acid diets, with growth data recorded over three weeks. Daily food intake records were kept. At the end of the three-week period, the rats were killed, the intestinal contents washed out and the carcasses frozen. The carcass analysis method was used to determine nitrogen retention during the experimental periods. The frozen carcasses were ground repeatedly and 2 to 3 gm of the homogenous pulp were taken in triplicate for nitrogen analysis by the Kjeldahl method. A group of rats (6 to 8), killed at the beginning of each experiment, was analyzed to determine initial body nitrogen.

The composition of the basal diet is given in table 1. Since the required levels of the essential amino acids had already been established[2] and it had been found that feeding a higher level of a mixture of these essential amino acids (thus increasing the total protein content of the ration) had no beneficial effect on growth rate, this established level for each of the essential amino acids was used and the total protein level was varied between 7.2 and 15% (N × 6.25) by altering the level of nonessential amino acid nitrogen added to the ration. In one experiment glycine served as the supplemental specific N source (table 2), whereas in the other, the mixture of nonessential amino acids patterned after those in casein (table 3) served as nonessential amino acid nitrogen source.

RESULTS AND DISCUSSION

Role of nonessential amino acid nitrogen. From the data in table 2 it is apparent that a significant depression in growth was obtained with increasing amounts of glycine added to the casein-amino acid diet to supply the nonspecific nitrogen requirement even though the 5.6% of casein is providing approximately 2.5% nonessential amino acid N × 6.25. As the protein level was increased by the addition of glycine, the growth rate de-

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[2] Rama Rao, P. B., V. C. Metta and B. C. Johnson 1957 The essential amino acid and protein requirements of the growing rat. Federation Proc., *16:* 397 (abstract).

TABLE 1

Essential amino acid mixture		Casein-amino acid diet	
	gm		*gm*
Amino acid		*Ingredient*	
L-Lysine·HCl	0.59	Casein[1]	5.6
L-Histidine·HCl monohydrate	0.05	Essential amino acid mixture	1.99
L-Tryptophan	0.09	Starch[2]	47.91
L-Isoleucine	0.17	Glucose[3]	10.0
L-Valine	0.17	Sucrose	10.0
L-Methionine	0.30	Lard	5.0
L-Threonine	0.28	Corn oil	5.0
L-Leucine	0.20	Wheat germ oil	0.5
L-Phenylalanine	0.14	Cod liver oil	1.5
Total	1.99	Vitaminized glucose[4]	5.0
		Mineral mixture 446[5]	4.0
		Sodium chloride	1.0
		Sodium bicarbonate	0.5
		Roughage[6]	2.0
		Total	100.00

[1] Labco, The Borden Company.
[2] Glycine or a mixture of nonessential amino acids (containing 12.3 gm DL-serine, 41.6 gm L-glutamic acid, 12.7 gm L-aspartic acid, 5.9 gm DL-alanine, 23.7 gm L-proline and 3.8 gm glycine) incorporated in diet at expense of starch as required.
[3] Cerelose, Corn Products Refining Company, New York.
[4] Vitaminized Cerelose. Rama Rao et al. ('59).
[5] Spector ('48).
[6] Wood Floc, Brown Company, Chicago.

TABLE 2

Effect of supplemental glycine on rat growth (19 days)

Diet[1]	Av. initial body weight	Glycine supplement	Av. weight gain[2]	Av. food intake	PER[3]
% protein	*gm*	*% diet*	*gm*	*gm*	
8.1	53	1.18	84 ± 2.8[4]	269	3.84 ± 0.11
10.2	53	2.89	80 ± 3.4	221	3.25 ± 0.09
12.1	51	4.60	77 ± 5.0	217	2.76 ± 0.21
14.0	53	6.32	68 ± 3.3	221	2.16 ± 0.13

[1] To the basal casein-amino acid diet (table 1) only glycine was added at graded levels to increase total protein ($N \times 6.25$) levels in diet.
[2] Average of 8 rats per group.
[3] $\frac{\text{Weight gain (gm)}}{\text{Protein intake (gm)}}$.
[4] Standard deviation of the mean.

creased from 4.4 gm per day to about 3.6 gm per day. Also the protein was used less efficiently, the protein efficiency ratio (PER) decreasing from 3.84 at the 8% protein level to 2.16 at 14% of protein. Growth depression due to glycine "toxicity" or "unpalatability" has been reported by Kinsey and Grant ('44), Hier et al. ('44), Arnstein ('54) and Harper et al. ('56). Rechcigl et al. ('57), in their studies on the net utilization of nonspecific nitrogen sources for the synthesis of nonessential amino acids, obtained their poorest growth response and net utilization when glycine and serine served as nitrogen sources. This agrees with the observations of Rose et al. ('49) and Birnbaum et al. ('57) that glycine is a poor source of "nonessential amino acid" nitrogen.

A mixture of nonessential amino acids patterned after casein (footnote 2, table 1) was tested as a source of nonessential amino acid nitrogen. The results given in table 3 show that no growth depression was obtained when the nonessential amino acid mixture was added in graded amounts so as to raise the protein level

TABLE 3

Minimum protein requirement for maximum growth and nitrogen retention

Casein-amino acid diet[1] (21 days)				
Protein (N × 6.25)	Av. weight gain	Av. food intake	Body protein gained[2] / Protein consumed (N × 6.25)	PER[3]
% diet	*gm/day*	*gm*		
A 7.2	4.2 ± 0.17[4]	279	0.69 ± 0.017	4.39 ± 0.15
B 7.9	4.7 ± 0.18	275	0.74 ± 0.018	4.59 ± 0.18
C 8.4	4.9 ± 0.20	271	0.76 ± 0.02	4.84 ± 0.06
D 9.0	5.1 ± 0.19	264	0.69 ± 0.021	4.94 ± 0.22
E 9.3	5.0 ± 0.21	261	0.72 ± 0.019	4.39 ± 0.11
F 10.0	5.1 ± 0.22	266	0.73 ± 0.020	4.37 ± 0.16
G 10.4	5.1 ± 0.19	263	0.68 ± 0.018	4.15 ± 0.10
H 12.8(7)[5]	5.2 ± 0.21	261	0.58 ± 0.02	3.16 ± 0.14
I 14.9(6)[5]	5.2 ± 0.20	258	0.47 ± 0.021	2.84 ± 0.19

[1] The basal ration contained 5.6% casein (5% protein [N × 6.25]) and the essential amino acids as given in table 1, providing an additional 1.6% protein (N × 6.25). Thus the total protein (N × 6.25) was 6.6%. Calculated quantities of the nonessential amino acid mixture (see footnote 2, table 1) were added to make up the varying protein (N × 6.25) levels given in column 1 (i.e., all N levels tested contain all the essential amino acids at the same requirement levels and the total N level is varied by changing the level of nonessential amino acids added).

[2] $\frac{\text{Nitrogen retained} \times 6.25}{\text{Nitrogen intake} \times 6.25}$; average of 6 rats per group.

[3] Protein efficiency ratio: $\frac{\text{Weight gain (gm)}}{\text{Total protein intake (gm)}}$.

[4] Standard deviation of the mean.

[5] Ten rats per group except as indicated.

to an equivalent of 14.9%. The growth rate remained at 5 gm per day with protein levels of 8.4 to 14.9%. Rose et al. ('48) obtained a maximum growth rate of 3.9 gm per day on a diet containing all 19 amino acids as compared with 3.2 gm per day with a diet containing the 10 essential amino acids plus glutamic acid, indicating a better response to a mixture of amino acids.

In comparing the growth rate of rats fed an all-amino-acid diet, as reported by Rose et al. ('48) and Rama Rao et al. ('60), with the growth rate obtained with this present casein-amino acid diet, it is obvious that the presence of 5% of protein (N × 6.25), supplied as casein (5.6%), gives improved growth rate. The primary objective in these studies is to establish the essential amino acid and total protein (N × 6.25) requirements of the rat to serve as a standard for nutritive evaluation of intact proteins, not amino acid rations. Thus the desire for a ration which would provide a good growth rate, necessitating an adequate food intake, prompted the use in these studies of a basal ration which provided part of the total protein (N × 6.25) as casein.

Protein level for maximum growth and nitrogen retention. The data in table 3 show that as the protein level is increased from 7.2 to 14.9% by the addition of the nonessential amino acid mixture, a maximum growth rate of 5 gm per day is obtained at 9% of protein. Mitchell and Beadles ('52), using defatted whole egg protein (laboratory-prepared), showed that the protein requirement for optimum growth (5 gm per day) of the rat under restricted conditions of feeding was about 12% of the diet and lower than the value of 16% reported by Hamilton ('39) with commercial whole egg protein. Block and Mitchell ('46) have reviewed the work proving that the requirement for a protein depends primarily on the content of its limiting essential amino acid. The casein-amino acid diets used in these studies provide all the essential amino acids at their minimum required levels (Rama Rao et al., '59).

Mitchell ('54) showed that a protein with a biological value of 100, correlates with a PER of about 5. The PER data

(table 3) show that a value of 4.9 is obtained with 9% of protein. Beyond the 9% protein level the efficiency of protein utilization decreases significantly, the PER decreasing to 2.84 at the 14.9% protein level ($P < 0.01$). Food intake data (table 3) show that at protein levels lower than 9% the rats consumed more diet and thus stored more fat, resulting in weight gains of 4.2 to 4.9 gm per day.

When the results are expressed as grams of body protein gained per gram of protein consumed (table 3), it is seen that at 10% of protein a value of 0.73 is obtained, with no significant change at lower protein levels. However, it falls off rapidly beyond the 10.4% protein level (0.58 at 12.8% and 0.47 at 14.9%, being significantly lower ($P < 0.01$) in both cases.

The growth data have been plotted in figure 1, the ascending line rising linearly for low protein percentages and the horizontal line for high protein percentages being fitted by the method of least squares. From the figure it can be seen that the maximum growth rate of 5.1 gm per day is reached at about 8.8% protein. Similarly, analysis of nitrogen retention data plotted in figure 1 reveals that a maximum retention of 143.7 mg of N per day is obtained at the 10% protein level. The fit is only fair, but there is no suggestion of any systematic departure, the deviation mean square being significant at the 5% level.

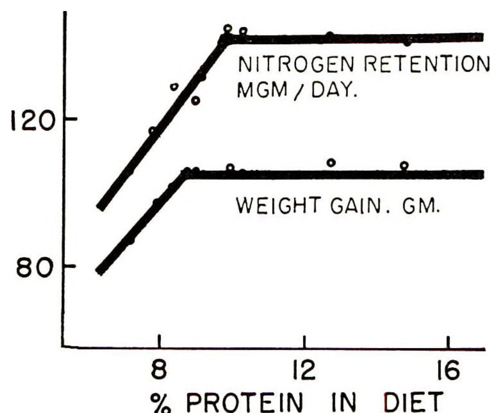

Fig. 1 Nitrogen retention vs. protein level ($N \times 6.25$); $Y = 13.17X + 12$; linear to 143.7 mg nitrogen retained at 10% protein; weight gain vs. protein level ($N \times 6.25$); $Y = 11.26X + 8.112$; linear to 107.2 gm at 8.8% protein.

SUMMARY

To determine protein requirement using a "balanced" protein, male weanling rats were fed casein-amino acid diets ad libitum for three weeks; these diets provided all the essential amino acids at the minimum required levels. The total protein levels tested ranged from 7 to 15%.

The minimum requirement of protein for maximum nitrogen retention was 10% of the diet, whereas for maximum growth it was only 8.8% of protein.

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An Investigation of the Essential Fatty Acid Activity of Some of the Geometrical Isomers of Unsaturated Fatty Acids[1]

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Linoleic acid was among the first fatty acids that Burr and Burr ('30) demonstrated to have essential fatty acid (EFA) activity. Their descriptions of the method for isolating the acid indicate that it was predominantly of the all *cis* form. Subsequent work by Holman ('51), Privett et al. ('55) and Holman and Aaes-Jorgensen ('56) demonstrated that the geometrical isomers of linoleic acid do not have EFA activity. It has been suggested that these geometrical isomers not only are inactive but also may interfere with the utilization of *cis,cis* linoleic acid (National Research Council, '58). However, this conclusion was based on one study using experimental groups of two animals each (Holman and Aaes-Jorgensen, '56).

In the study reported here, the EFA activity of the geometrical isomers of linoleic acid either when fed alone or in a mixture with the all *cis* form was determined. In confirmation of earlier work, the *cis,trans* and *trans,trans* isomers of linoleic acid were found to have no EFA activity. In addition, it was found that they do not interfere with the activity of the all *cis* form.

PROCEDURE

A diet free of essential fatty acids was fed to 130 weanling male rats, housed in individual cages. The percentage composition of the diet was: casein, vitamin-free, 20.0; sucrose, 70.0; salt mixture, U.S.P. 14, 4.0; cellu flour, 3.0;[2] hydrogenated coconut oil, 3.0. The usual water-soluble vitamins were added to the diet with the sucrose, and the fat-soluble vitamins were dissolved in the coconut oil.[3] This diet was fed to the animals throughout the experiment.

After following this regimen for approximately 12 weeks, the animals showed no further gain in body weight for 10 days. At this time the animals were separated into 13 groups of 10 animals each. Distribution among groups was such that each group had approximately the same average and median body weight. For a subsequent 4-week period the diet of the animals was supplemented daily with either 50 or 100 mg of the ethyl esters of various fatty acids. The negative control group received 50 mg of hydrogenated coconut oil. During this period, body weight was determined at weekly intervals.

The supplements administered to the animals and the analytical values obtained with these are given in table 1. The *cis, cis* linoleate was isolated from safflower seed oil. The fatty acids of this oil were converted to the ethyl esters and the linoleate was isolated by removing the saturated and monounsaturated acids by urea adduction. A portion of the isolated linoleate was isomerized with sulfur dioxide at 200°C. The *cis,trans* and *trans,trans* isomers were separately isolated by re-

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[1] By definition, linoleic acid is the all *cis* isomer of 9,12-octadecadienoic acid. In this paper the terms *cis,cis*, *cis,trans*, or *trans,trans* linoleic acid will be used to distinguish the various isomers. The *cis,trans* linoleic acid used was probably a mixture in equal proportions of the *cis,trans* and *trans,cis* isomers.

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

[3] Each 1000 gm of diet contained the following vitamins in mg/kg of diet: menadione, 3; thiamine·HCl, 4; riboflavin, 5; niacin, 20; Ca pantothenate, 20; folic acid, 0.25; pyridoxine, 4; vitamin B_{12}, 0.15; choline chloride, 3000; inositol, 2000; ascorbic acid, 100; *p*-aminobenzoic acid, 100; biotin, 0.25; vitamin A acetate, 0.52; calciferol, 0.0075; α-tocopheryl acetate, 30.

TABLE 1

Analytical values obtained using various ethyl ester supplements

		Iodine value	Fatty acid composition, spectrophotometric					*Trans* bonds/molecule, infrared
			Saturated	Monoenoic	Dienoic	Trienoic	Conjugated diene	
Hydrogenated coconut oil	Observed	1.1	98.8	1.2	0.0	0.0	0.0	0.0
cis, cis Linoleate	Observed	163.1	0.0	0.0	100.7	0.0	0.0	0.0
	Theory	164.5	0.0	0.0	100.0	0.0	0.0	0.0
cis,trans Linoleate	Observed	161.8	—	—	—	—	—	1.0
	Theory	164.5	—	—	—	—	—	1.0
trans,trans Linoleate	Observed	162.4	—	—	—	—	—	1.9
	Theory	164.5	—	—	—	—	—	2.0
Oleate	Observed	82.5	0.0	100.1	0.3	0.0	0.0	0.0
	Theory	81.8	0.0	100.0	0.0	0.0	0.0	0.0
Elaidate	Observed	82.3	—	—	—	—	—	1.0
	Theory	81.8	—	—	—	—	—	1.0

peated low-temperature crystallizations of an acetone solution of this mixture of the linoleate isomers. The analytical values shown in table 1 demonstrate the purity of the linoleic acid and its isomers.

The oleic acid was obtained from olive oil. The fatty acids of this oil were converted to the ethyl esters and the saturated esters removed by treatment with urea. Ethyl oleate was isolated from an acetone solution of the non-adduct-forming fraction by repeated low-temperature crystallization. A portion of the isolated oleate was isomerized with sulfur dioxide at 200°C. Ethyl elaidate was isolated from this by crystallization from acetone. The analytical values obtained from these monoenoic acids indicate them to be of high purity (table 1).

RESULTS

The average cumulative gains in body weight for the various groups of animals after 4 weeks of supplementation are given in table 2. Although the body weights of the animals had apparently stabilized at the time they were assigned to the various groups, a further slight gain was observed during the period of supplementation which is not attributable to the feeding of essential fatty acids. This is demonstrated by the group fed the hydrogenated coconut oil.

Supplementation with *cis,cis* linoleate alone resulted in a marked increase in body weight. On the other hand, supplementation with *cis,trans* or *trans,trans* linoleate, oleate, or elaidate did not result in a gain in body weight over that of the negative controls which received the hydrogenated coconut oil. However, the animals given these latter supplements did not gain significantly less weight than the negative controls.

When oleic or elaidic acid or these various isomers of linoleic acid were administered to the animals along with the *cis, cis* linoleate, the gain in body weight was the same as that when the *cis,cis* linoleate alone was fed. Thus, the monounsaturated acids and these isomers of linoleic acid did not interfere with the utilization of the *cis,cis* linoleate, given as a supplement, nor increase the requirement for essential fatty acids.

TABLE 2

The average cumulative gain in body weight of EFA-deficient rats after 4 weeks of daily supplementation with 50 or 100 mg of the ethyl esters of various fatty acids

Supplement	Level of supplement	Cumulative weight gain[1]
	mg/day	*gm*
Hydrogenated coconut oil	50	25.2 ± 3.8[2]
cis,cis Linoleate	50	46.5 ± 4.0
cis,trans Linoleate	50	26.7 ± 3.9
trans,trans Linoleate	50	30.7 ± 1.3
cis,trans Linoleate, plus *trans,trans* linoleate	25, 25	19.9 ± 4.7
Oleate	50	26.9 ± 3.3
Elaidate	50	18.3 ± 5.5
Supplement of 50 mg of *cis,cis* linoleate per day, plus that listed for each group		
cis,trans Linoleate	50	46.0 ± 2.6
trans,trans Linoleate	50	46.3 ± 4.1
cis,trans Linoleate, plus *trans,trans* linoleate	25, 25	47.6 ± 2.3
Oleate	50	48.8 ± 2.2
Elaidate	50	45.7 ± 3.5

[1] The minimum significant difference between the means of any two groups as determined by the method of Tukey ('52) is 16.7 gm at a confidence level of 0.05.

[2] Standard error of the mean.

DISCUSSION

Many of the reports in the literature on the effect of various types of dietary fat on EFA deficiency are apparently conflicting. A study of these, however, will show that the apparent conflict in many instances is due to poor characterization of the test materials. This insufficient characterization resulted from a lack of suitable analytical techniques at the time the earlier studies were carried out or from a failure by later workers to apply the analytical methods which had become available. Where well-characterized test materials have been used these apparent conflicts have not arisen.

Funch et al. ('59) and Aaes-Jorgensen et al. ('56) concluded that hydrogenated arachis oil accentuated EFA-deficiency symptoms and that this "deleterious effect of hydrogenated arachis oil could be partly due to the presence of isomers of the unsaturated fatty acids formed during hydrogenation." However, melting point was the only character of this fat presented. From this alone, it is impossible to judge the type or level of unsaturated fatty acid isomers that might be present. These results are in conflict with those of Deuel et al. ('51) who observed, in general, excellent agreement between the values for the linoleic acid content of a hydrogenated fat as determined by the spectrophotometric method and by biological assay. If the isomers of the unsaturated fatty acids had an anti-EFA activity the values obtained by the spectrophotometric method should have been higher than those obtained by the biological assay. On the contrary, in the case of the one fat which did not yield good agreement between the two methods, the biological assay gave the higher value for EFA content.

Experiments in which isolated fatty acids of known composition or well-characterized fats were used, now present a consistent pattern as to the EFA activity of the isomers of the unsaturated fatty acids. The experiments of Alfin-Slater et al. ('57) show clearly that the *trans* isomers of oleic acid formed as the result of hydrogenation do not have EFA activity. On the other hand, they do not interfere with the utilization of essential fatty acids. The experiments reported here demonstrate that the same is true for elaidic acid formed by isomerization with sulfur dioxide.

Early work by Holman ('51) could be interpreted as meaning that conjugated linoleic acid had an anti-EFA activity. However, a more recent report from his

laboratory by Aes-Jorgensen ('58) demonstrates that conjugated *cis,trans* or *trans, trans* linoleate and eleosterate do not have any EFA activity but, like the geometrical isomers of oleate, they do not have anti-EFA action.

In 1951, Holman ('51) reported that *trans,trans* linoleate had no EFA activity. Subsequent work (Privett et al., '55) demonstrated that this was true also for *cis, trans* linoleate. The results reported in this paper confirm that the geometrical isomers of linoleic acid do not have any EFA activity.

In contrast with the results reported here, Holman and Aaes-Jorgensen ('56) concluded that the geometrical isomers of linoleic had an anti-EFA activity. However, because of the considerable variation between animals in their response to EFA deficiency and the fact that Holman and Aaes-Jorgensen used only two animals per group in their studies, the experiment reported here was carried out using groups of sufficient size to permit statistical treatment of the data. The necessity for using groups of adequate size was observed in the present study. For example, the range in cumulative gain in body weight for the animals receiving only the elaidate was from minus 23 to plus 35 gm; whereas, the range for the animals receiving oleate was from plus 10 to plus 43 gm. If groups of only two animals each were used and chance had dictated that these were the 4 animals selected, one could have been led to conclude that oleate was superior to elaidate; whereas, with groups of 10 animals it is apparent that no real difference exists between these two fatty acids. Since the conclusion of the Committee on Fats in Human Nutrition of the National Research Council ('58) that " . . . trans isomers of linoleic acid may accentuate or hasten essential fatty acid deficiency symptoms if the diet is extremely low in linoleic or arachidonic acid . . . " is based on this study of Holman and Aaes-Jorgensen, their conclusion may need to be modified in view of the results reported here.

Greenberg et al. ('50) showed that linolenic acid by itself had no EFA activity. However, if linoleic acid was present also, it "sparked" the utilization of linolenic acid and the activity of linolenic acid was then equal to that of linoleic acid. Privett et al. ('55) concluded from their studies that *cis,trans* linoleate could not "spark" the utilization of all *cis* linolenate as judged by dermal symptoms and water intake. However, the *cis,trans* isomer of linoleate plus all *cis* linolenate gave greater growth than either alone. Thus, it appears probable, although not absolutely established, that the geometrical isomers of linoleic acid cannot "spark" linolenic acid so that it can function as an EFA.

The addition of completely hydrogenated coconut oil to an otherwise fat-free diet speeds up the development of an EFA deficiency (Deuel et al., '51). Supplementation of such diets with adequate amounts of *cis,cis* linoleate allows the resumption of growth. Moreover, the hydrogenated coconut oil does not interfere with the utilization of the supplementary EFA (Deuel et al., '55). This ability to speed-up the development of EFA deficiency is shared by a number of fatty acids; namely, palmitate, conjugated *cis,trans* or *trans,trans* linoleate, and eleosterate (Aaes-Jorgensen, '58). But here, too, it was found that these acids do not have an anti-EFA activity. It appears that the presence in the diet of fat having no EFA activity speeds up the turnover of the body's store of essential fatty acids but does not affect the utilization of essential fatty acids when they are added to such diets.

The status of our knowledge with respect to the EFA activity of various fatty acid isomers can be summarized as follows: (1) dietary fatty acid not possessing EFA activity will hasten the development of deficiency symptoms; (2) the geometric isomers of linoleic acid and conjugated linoleic acid do not have EFA activity nor do they have an anti-EFA activity; (3) the geometric isomer of oleic acid whether formed by hydrogenation or sulfur dioxide isomerization, like oleic acid, does not have EFA activity; (4) the "sparking" of linolenate by linoleate, thus far, is unique to this one acid and does not apply to monoenoic acids nor probably to the isomers of linoleic acid.

It has been reported (Evans et al., '34; Panos and Finerty, '54; Aaes-Jorgensen et al., '56; Funch et al., '59) that EFA de-

ficiency results in an interference with spermatogenesis. This was confirmed by Holman and Aaes-Jorgensen ('56) who stated further that the condition was worsened by the presence of linoleate isomers in the diet. However, in subsequent publications (Aaes-Jorgensen, '58; Aaes-Jorgensen and Holman, '58; Aaes-Jorgensen et al., '58) normal spermatogenesis was observed in EFA-deficient animals. In the study reported here the testes of many of the animals were examined and all showed normal spermatogenesis. In view of these conflicting reports, it remains a moot point whether EFA deficiency results in an impaired spermatogenesis.

SUMMARY

Essential fatty acid (EFA) deficiency was developed in weanling male rats by feeding them a diet deficient in essential fatty acids for approximately 12 weeks. The animals were then divided into groups and given supplements of *cis,cis* linoleate, *cis,trans* linoleate, *trans,trans* linoleate, oleate, or elaidate either alone or in addition to *cis,cis* linoleate. On the basis of gain in body weight it is concluded that only *cis,cis* linoleate possesses EFA activity. However, the inactive acids did not interfere with the utilization of dietary *cis,cis* linoleate.

Spermatogenesis was normal in the EFA-deficient animals.

The status of our knowledge of the EFA activity of the geometrical isomers of unsaturated fatty acids is reviewed.

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Observations on the Influence of Vitamin B_{12} and Folic Acid on Protein Utilization in the Growing Rat[1]

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Since the recognition of vitamin B_{12} as a major constituent of animal protein factor, several reports have emphasized improvement in the utilization of low-quality vegetable proteins by supplementation with this vitamin (Marfatia and Sreenivasan, '51; Baliga et al., '54; Sure, '54, '57). The effectiveness of vitamin B_{12} is particularly marked at low-protein intakes (Hartman et al., '49; Luecke, '49; Emerson, '49) and may vary with different vegetable proteins and also with the animal species. (Wokes and Picard, '56). A number of workers have established the existence of relationships between vitamin B_{12}, folic acid (PGA), methionine and choline in the rat, mouse, chick and certain other species (Bennett, '50; Schaefer et al., '50; Schaefer and Knowles, '51; Stekol et al., '52; Jukes and Stokstad, '52; Sauberlich, '59). An interdependence of vitamin B_{12} and PGA may also be inferred from the common and as yet undissociable functions of these vitamins in several metabolic processes and also from observations on their mutual potentiation (Sreenivasan, '51; Girdwood, '59; Fatterpaker et al., '55; Narayanan et al., '56; Ellis et al., '59). The object of the present investigation was to assess the influence of vitamin B_{12} and PGA, in the growing rat, on protein utilization using varying levels of dietary protein.

EXPERIMENTAL

Young, male Wistar rats of approximately 50-gm weight were used in the trials with peanut protein and casein. The animals were initially depleted of their vitamin B_{12} and PGA reserves by maintenance on a deficient, iodo-casein diet consisting of (in gm per 100 gm of diet): vitamin-free casein, 10; iodinated casein,[3] 0.15; arachis oil, 6; shark liver oil, 2; sucrose, 9.85; corn starch, 68; and salt mixture (U. S. P. no. 14), 4. The sucrose provided the following vitamin levels (in mg per kg of diet): thiamine·HCl, 6; riboflavin, 10; Ca pantothenate, 20; pyridoxine·HCl, 6; biotin, 1; nicotinic acid, 30; choline chloride, 500; and inositol, 500. The arachis oil carried supplements of α-tocopherol and of 2-methyl-1, 4-naphthoquinone at levels of 50 mg and 10 mg, respectively, per kilogram of diet. The vitamin levels provided were considered adequate for the hyperthyroid condition.

At the end of 4 weeks, the animals were divided into groups of 8. One group was replaced on the original iodo-casein diet modified by the omission of iodinated casein and inclusion of succinyl sulphathiazole at a 2% level (with appropriate adjustment of the starch content). A second group received the protein (vitamin-free casein) at an 18% level in the modified ration, the extra protein addition being made at the expense of corn starch. Two similar groups were fed with defatted, hot alcohol-extracted peanut meal at 10 and 18% protein levels. There were corresponding groups in each case receiving supplements of vitamin B_{12} and PGA at levels of 150 μg and 5 mg, respectively, per kg of diet. There were 4 groups in addition to these 8, fed the casein or peanut protein

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[3] Iodinated casein, Protomone, Cerophyl Laboratories, Kansas City, Missouri.

at a 10% level and receiving supplements as above either of vitamin B_{12} or PGA.

The animals were maintained in individual, suspended cages with 1/2-inch mesh screen bottoms. The average initial weight for the groups (around 86 gm) did not differ by more than 2 gm. The rats were weighed twice weekly and fed ad libitum, with food intake recorded.

In the trials with corn-peanut meal, male weanling rats weighing from 40 to 50 gm were used, receiving the rations described in table 4. By analysis the protein content of the basal ration was 30% and with appropriate additions of starch, where necessary, this level was maintained in all other rations. The basal ration was similar in composition to that developed in this laboratory for the production of vitamin B_{12} deficiency in rats and mice (Fatterpaker et al., '59), and included succinyl sulphathiazole. Its percentage composition was as follows: defatted, alcohol-extracted peanut meal, 46; corn meal, 44; vitaminized arachis oil, 5; vitaminized sucrose, 1; salts mixture (no. 4, Hegsted et al., '41), 2; and succinyl sulphathiazole, 2. The vitamin levels provided by sucrose and arachis oil corresponded to those used in the iodinated-casein diet in the previous experiment. Each rat was given orally at the beginning of the experiment and each week thereafter two drops of shark liver oil to provide 300 I.U. of vitamin A and 4 I.U. of vitamin D. During the experimental 6-week period the animals had free access to food and water. At three weeks of feeding, nitrogen retention studies with 4 animals per group were made during three equally spaced 4-day periods in the 4th, 5th and final week. Individual, round metabolism cages were used, urine and feces being collected before the morning feeding. Urine was stored with the addition of a few drops of sulphuric acid and toluene. Feces were dried at 80°C for 24 hours and weighed. The nitrogen content of the excreta pooled for each 4-day period was determined by the Kjeldahl method. Growth rate and food intake were recorded throughout the entire period.

The rats were dissected under ether anesthesia. Livers were quickly excised, chilled and made into 20% homogenates with ice-cold distilled water. Total liver nitrogen was determined by the micro-Kjeldahl procedure of Umbreit ('46). Total liver fat determination was made according to the method of Sperry ('54). Protein-free metaphosphoric acid extracts of the tissue were analyzed for non-protein sulphydryl by the nitroprusside method of Grunert and Phillips ('51) standardized against glutathione. In the trials with casein and peanut protein, livers were additionally analyzed for vitamin B_{12} and PGA, and blood was also obtained in this experiment for determination of serum vitamin B_{12}. Blood was drawn from the hepatic portal vein prior to excision of the liver and was allowed to clot at 37°C for an hour and later centrifuged in cold to separate the serum. Total vitamin B_{12} in the latter was determined by the method of Ross ('52) using the *bacillaris* strain of *Euglena gracilis*. Portions of liver homogenates were incubated at 37° for 12 hours under toluene with papain (25 mg/gm of fresh liver) in 0.1 M acetate buffer of pH 4.6. The samples were then analyzed for vitamin B_{12} using *E. gracilis*, according to the method of Hoff-Jorgensen ('54). Separate portions of the homogenates were autolyzed in 0.1 M phosphate buffer of pH 7.2 under toluene at 37° for 12 hours and assayed with *Streptococcus faecalis R* for PGA as described by Mitbander and Sreenivasan ('54).

RESULTS

Administration of vitamin B_{12}, and especially of PGA, or of the two simultaneously was associated with a marked increase in food intake in both peanut protein- and casein-fed animals (table 1). The efficiency ratio with peanut protein was lower than with casein at both low- (0.54, $P < 0.01$) and high- (0.47, $P < 0.01$) protein intakes. The 10% protein diet with vitamin B_{12} or PGA resulted in better utilization of peanut protein, with marked improvement when both vitamins were fed simultaneously, irrespective of the amount of protein consumed. The gains in body weight followed a similar trend although PGA was somewhat more effective than vitamin B_{12} in reducing the difference in the growth rate due to protein quality.

With low-protein diets the liver nitrogen value was higher with casein (23.6 mg/

TABLE 1

Summary of individual and combined effects of dietary vitamin B_{12} and PGA on the efficiency of peanut protein and casein utilization by the rat[1]

Diet description		Peanut protein-fed rats			Casein-fed rats[2]		
Protein content	Supplements	Protein consumed	Weight gained	Protein efficiency ratio[3]	Protein consumed	Weight gained	Protein efficiency ratio[3]
%		gm	gm		gm	gm	
10	None	20.1 ± 1.2[4]	13.7 ± 2.0	0.68 ± 0.03	18.0 ± 0.8	$22.0 \pm 2.4^{++}$	$1.22 \pm 0.11^{++}$
10	Vitamin B_{12}	25.0 ± 1.2	26.5 ± 2.1	1.06 ± 0.09	26.5 ± 1.4	$39.5 \pm 3.3^{++}$	$1.49 \pm 0.10^{++}$
10	PGA	31.4 ± 1.3	34.8 ± 3.1	1.11 ± 0.08	29.0 ± 1.3	$43.8 \pm 3.0^{+}$	$1.51 \pm 0.10^{++}$
10	Vitamin B_{12} + PGA	31.8 ± 1.8	53.4 ± 3.4	1.68 ± 0.12	33.5 ± 1.8	61.0 ± 2.8	1.82 ± 0.07
18	None	41.6 ± 2.1	27.9 ± 2.8	0.67 ± 0.08	37.4 ± 1.6	$42.6 \pm 3.8^{++}$	$1.14 \pm 0.08^{++}$
18	Vitamin B_{12} + PGA	54.3 ± 3.2	83.1 ± 4.6	1.53 ± 0.14	58.6 ± 3.4	$100.2 \pm 6.2^{+}$	1.71 ± 0.09

[1] Data obtained over a 5-week period of maintenance of vitamin B_{12}- and folic acid-deficient animals on deficient, peanut protein and casein diets with and without supplements of vitamin B_{12} (B_{12}, 150 μg/kg of diet) and folic acid (PGA, 5 mg/kg of diet).

[2] Statistically significant differences from corresponding values of peanut protein-fed animals are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$.

[3] Grams gain in body weight per gram of protein consumed.

[4] Standard error of the mean.

TABLE 2

Effects of vitamin B_{12} and PGA on liver composition with peanut protein and casein diets[1]

Diet description		Liver analysis of peanut protein-fed rats			Liver analysis of casein-fed rats[2]		
Protein content	Supplements[3]	Total nitrogen	Total fat	Non-protein sulphydryl	Total nitrogen	Total fat	Non-protein sulphydryl
%		mg/gm	mg/gm	mg/gm	mg/gm	mg/gm	mg/gm
10	None	18.8 ± 0.5	43.4 ± 1.8	0.61 ± 0.07	$23.6 \pm 0.7^{++}$	$34.2 \pm 2.1^{++}$	$1.01 \pm 0.06^{++}$
10	Vitamin B_{12}	24.2 ± 1.2	32.2 ± 2.7	0.84 ± 0.06	$28.2 \pm 0.9^{+}$	$25.9 \pm 1.0^{++}$	$1.37 \pm 0.06^{++}$
10	PGA	20.8 ± 1.4	35.4 ± 1.9	0.65 ± 0.09	$27.3 \pm 0.8^{++}$	$28.6 \pm 2.6^{++}$	$1.02 \pm 0.05^{++}$
10	Vitamin B_{12} + PGA	27.1 ± 1.4	28.2 ± 2.1	0.95 ± 0.04	29.4 ± 1.1	$22.7 \pm 2.0^{++}$	$1.52 \pm 0.04^{++}$
18	None	19.4 ± 0.8	35.7 ± 1.8	0.88 ± 0.07	$26.2 \pm 0.4^{++}$	$25.8 \pm 2.9^{++}$	$1.51 \pm 0.05^{++}$
18	Vitamin B_{12} + PGA	27.3 ± 1.0	23.5 ± 1.6	1.88 ± 0.08	$31.2 \pm 0.7^{++}$	21.8 ± 1.4	$1.85 \pm 0.07^{++}$

[1] Values are on wet basis and include the standard error of the mean.

[2] Statistically significant differences from corresponding values of the peanut protein-fed are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$.

[3] B_{12} = vitamin B_{12}, 150 μg/kg of diet; PGA = folic acid, 5 mg/kg of diet.

gm) than with peanut protein (18.8 mg/gm) (table 2). Significant reduction of this difference was not obtained with vitamin B_{12} or PGA alone, but only with their simultaneous provision. With increased intake of protein there was a significant gain in liver nitrogen in the casein-fed animals (2.6 mg/gm, $P < 0.05$) but not in the peanut protein-fed group (0.6 mg/gm, $P > 0.1$). As a result, using the high-protein diets the difference in nitrogen values between the two groups was magnified (6.8 mg/gm, $P < 0.01$) and although with vitamin B_{12} and PGA this was considerably rectified, a significant difference still prevailed (3.9 mg/gm, $P < 0.01$). Livers of the doubly deficient animals showed marked fatty infiltration especially from those from rats fed low-protein diets and with peanut protein. Vitamin B_{12} or PGA brought about appreciable reductions in liver fat, the two vitamins together causing still further depression. These reductions were in every instance attended by a significant diminution of the effect due to protein quality. With a high-protein regimen the lipotropic effects of the vitamins extended to bringing about a near equalization of the liver fat contents in the casein and peanut protein groups. The liver content of non-protein sulphydryl was appreciably lower in the peanut protein-fed rats than in those receiving casein. Administration of vitamin B_{12} alone caused a significant improvement, whereas PGA was effective only in presence of vitamin B_{12}, especially in low-protein rations.

Observations on serum and liver vitamin B_{12} and liver PGA are summarized in table 3. The depletion of the liver reserves of the vitamins in the doubly deficient animal was greater in the peanut protein than in the casein-fed animals. A similar, though less marked trend, was seen in the serum concentration of vitamin B_{12}. However, in the replete groups the serum level of vitamin B_{12} was considerably higher in the peanut protein than in the casein-fed rats although the liver levels of the vitamins were again lower in the former. Such reductions of liver vitamin B_{12} and PGA levels, with elevation of serum vitamin B_{12} concentration, also accompanied low-protein intakes. In general, the administration of either vitamin B_{12} or of PGA to the doubly deficient animal raised the liver concentration of the other vitamin (also serum concentration of vitamin B_{12} when PGA was fed).

The effects of partial replacement of peanut protein by egg albumen in the basal corn-peanut meal on the response to vitamin B_{12} and PGA are summarized in tables 4 and 5. Although the rats were fed unrestrictedly, no significant differences in food intakes were observed as a result of vitamins or egg albumen supplementation. Either supplement effected higher nitrogen retention and improved the growth rate. The effectiveness of the vitamins was markedly reduced in the egg albumen-containing ration. This trend was supported by the liver analyses (table 5). With supplements of egg albumen or vitamin B_{12} and PGA there resulted significant ($P < 0.01$) increases in liver nitrogen and non-protein sulphydryl and a decrease ($P < 0.01$) in liver fat. With the incorporation of egg albumen in the diet, vitamin B_{12} and PGA supplements effected only a small, although significant ($P < 0.05$), increase in non-protein sulphydryl, nitrogen and fat contents remaining unchanged.

DISCUSSION

According to Baernstein ('37) and Randoin and Boisselot ('43), the proteins of peanut are approximately equal to casein in promoting the growth of rats when fed at the 20% level. Peanut protein is deficient in sulphur-containing amino acids, particularly methionine (Grau, '46). This, while accounting for the low liver non-protein sulphydryl using peanut protein diets, would suggest that the growth-promoting effect observed with vitamin B_{12} and PGA is due, at least in part, to the known methionine-sparing effect of the vitamins. Both from the growth data as well as the liver analysis this effect would appear to be an additive one. Particularly it was seen that the liver nitrogen content of peanut protein-fed rats improved only when both vitamins were supplied. The lipotropic effect of the vitamins also appeared to be additive and unrelated to increased food intake or growth. The observation that this effect is not well-marked with casein to the same extent that it is with peanut protein under identical con-

TABLE 3

Serum and liver vitamin B_{12} and liver PGA[1]

Diet description		Peanut protein-fed rats			Casein-fed rats[2]		
Protein content	Supplements[3]	Serum vitamin B_{12}	Liver vitamin B_{12}	Liver PGA	Serum vitamin B_{12}	Liver vitamin B_{12}	Liver PGA
%		μμg/ml	mμg/gm	μg/gm	μμg/ml	mμg/gm	μg/gm
10	None	82 ± 20	9.0 ± 1.8	1.01 ± 0.32	134 ± 22++	25.9 ± 6.7++	2.16 ± 0.21++
10	Vitamin B_{12}	722 ± 28	54.6 ± 9.8	1.84 ± 0.63	636 ± 28++	74.2 ± 7.9+	2.82 ± 0.19++
10	PGA	145 ± 16	15.7 ± 4.2	3.72 ± 0.41	163 ± 14	38.6 ± 4.2++	4.98 ± 0.31++
10	Vitamin B_{12} + PGA	1320 ± 42	62.4 ± 6.6	4.31 ± 0.20	850 ± 46++	92.6 ± 7.4++	5.34 ± 0.26++
18	None	40 ± 8	14.5 ± 7.9	2.10 ± 0.38	84 ± 18++	35.9 ± 6.2++	2.84 ± 0.32+
18	Vitamin B_{12} + PGA	976 ± 36	80.7 ± 8.4	5.32 ± 0.22	625 ± 34++	121.8 ± 9.8++	6.30 ± 0.29++

[1] Mean values and their standard errors; liver composition is on wet basis.
[2] Statistically significant differences from corresponding values of peanut protein-fed are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$.
[3] Vitamin B_{12}, 150 μg/kg of diet; folic acid (PGA), 5 mg/kg of diet.

TABLE 4

Nitrogen balance and growth rate of rats fed the basal corn-peanut meal ration with and without supplements of egg albumen and vitamin B_{12} and PGA[1]

Supplements to basal diet[2]	Nitrogen retention[3]		Total food intake in 6 weeks	Weight gained in 6 weeks
	Food intake	Nitrogen balance		
	gm/day	mg/day	gm	gm
None	8.66 ± 0.18	176 ± 11.3	348 ± 15	55.8 ± 4.1
Vitamin B_{12} + PGA	9.01 ± 0.24	215 ± 8.8++	357 ± 7	88.9 ± 3.5++
Egg albumen[4]	8.62 ± 0.11	221 ± 9.7	353 ± 14	81.0 ± 4.5
Egg albumen + B_{12} + PGA	8.78 ± 0.13	240 ± 6.9+	365 ± 12	101.2 ± 5.6++

[1] Mean values and their standard errors; statistically significant effects due to vitamin B_{12} and PGA supplementation are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$. There were 6 animals per series.
[2] B_{12} = 150 μg of vitamin B_{12}/kg of diet; PGA = 5 mg of folic acid/kg of diet; where used, egg albumen was at 3% level in diet and replaced an equal amount of peanut protein; protein content of all diets was by analysis 30%.
[3] Summary of data obtained with 4 animals/group over three separate periods of 4 days each.
[4] Egg albumen, Nutritional Biochemicals Corporation, Cleveland.

TABLE 5
Liver nitrogen, fat and non-protein sulphydryl in rats on the basal corn-peanut meal ration with and without supplements of egg albumen and vitamin B_{12} and PGA[1]

Supplements to basal diet[2]	Total nitrogen	Total fat	Non-protein sulphydryl
	mg/gm	*mg/gm*	*mg/gm*
None	23.9 ± 0.5	31.6 ± 1.3	0.96 ± 0.07
Vitamin B_{12} + PGA	$29.5 \pm 0.6^{++}$	$25.2 \pm 1.6^{++}$	$1.28 \pm 0.04^{++}$
Egg albumen	29.7 ± 0.5	23.8 ± 2.1	1.34 ± 0.06
Egg albumen + vitamin B_{12} + PGA	31.1 ± 0.7	20.9 ± 1.8	$1.54 \pm 0.03^{+}$

[1] Values are on wet basis and include the standard error of the mean; statistically significant differences due to vitamin B_{12} and PGA supplements are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$.

[2] See footnote 2, table 4.

ditions of protein intake, but presumably with a higher methionine intake, would suggest that the critical factor was the role of vitamin B_{12} and PGA in the synthesis and transfer of labile methyl groups.

Examination of the data reveals an inverse relationship between liver fat content and the level of the vitamins in this tissue. The possibility that the degree of fat accumulation in liver may influence its retention of the vitamins may, therefore, be considered. In the event of a free supply of the vitamins in the diet, greater concentrations of these may be found in plasma and in urine when the diet is based on peanut protein than when based on casein.

It is of interest to recall the observations of Fox and his associates who found that raising the fat level of a corn-soybean meal diet from 3 to 22% increased the severity of vitamin B_{12} deficiency (as assessed by growth and mortality rates) in non-depleted chicks (Fox et al., '54) and elevated the vitamin B_{12} requirement (Fox et al., '56); this high vitamin requirement could be eliminated by supplemental methionine (Fox et al., '57, '59). Although in these experiments the high level of dietary fat, apparently, did not cause depletion of liver store of vitamin B_{12}, or increase the liver fat content, it lowered the ability of the tissue to retain any administered vitamin B_{12} (Fox et al., '56, '59). In this laboratory it has been observed that with rats fed 18% casein or wheat gluten diets providing minimal or optimal levels of B vitamins, increasing the dietary level of fat from 8 to 15% increased the concentration of liver lipids by about 20 to 34%.[4]

The retention of vitamin B_{12}, PGA and, probably, also other B factors in the liver tissue could, therefore, be considered as being influenced, directly or otherwise, by the degree of fat deposition in the tissue. Such an effect may also explain the highly depleted state of the doubly-deficient animals fed peanut protein diets.

The observation that the administration of either vitamin B_{12} or PGA to the doubly-deficient animal serves to raise the liver concentration of the other vitamin, substantiates earlier reports (*vide infra*) on their mutual potentiation.

The absence of any significant effect of vitamin B_{12} and PGA on food intake in experiments with corn-peanut meal may be due to the high-protein content of the diet (30%). The data in this experiment are indicative of a greater retention of dietary nitrogen, improved growth rate and liver nitrogen and non-protein sulphydryl contents as a result of partial replacement of dietary protein by egg albumen. Since similar improvements could also be brought about with supplements of vitamin B_{12} and PGA, presumably through a sparing action on methionine, it would seem that the primary deficiency in the basal diet was that of sulphur-amino acids; the liver non-protein sulphydryl content was low with this diet. Further, the effectiveness of vitamin B_{12} and PGA was reduced with the increase in the average quality of dietary protein brought about by egg albumen supplementation. Comparative studies of protein quality and utilization have been reported by Fatterpaker et al. ('59) and Marfatia and Sreenivasan ('60).

In rats deficient in vitamin B_{12} the growth-promoting effect of the vitamin has

[4] T. Balakrishna Rao, U. Marfatia and A. Sreenivasan, unpublished data.

been attributed to increased deposition of fat rather than to synthesis of new protein (Arnstein, '55). Henry and Kon ('56) further suggest that the effect of vitamin B_{12} on protein efficiency may be discounted since the method hinges on the composition of weight gain in relation to protein intake. A reappraisal of these ideas may be necessitated if observations reported recently (Wagle et al., '58) on the role of vitamin B_{12} in protein synthesis are corroborated.

SUMMARY

Young rats depleted of their vitamin B_{12} and folic acid reserves were used to study the influence of these vitamins on the efficiency of protein utilization from peanut meal and casein diets at two protein levels.

The low efficiency of peanut protein in comparison with casein was improved with supplements either of vitamin B_{12} or folic acid. For optimal effects supplementation with both vitamins was essential.

Livers of the doubly-deficient animals showed marked fatty infiltration, with low nitrogen and non-protein sulphydryl content, especially with low-protein diets and with peanut protein.

Comparable groups of peanut protein- and casein-fed animals exhibited differences in liver composition with respect to nitrogen, fat and non-protein sulphydryl.

In separate experiments with weanling rats fed a vitamin B_{12}- and PGA-deficient, corn-peanut meal diet (30% protein) for 6 weeks of unrestricted feeding, replacing 3% of peanut protein with egg albumen, increased nitrogen retention, improved the growth rate and liver content of nitrogen and non-protein sulphydryl and effected a decrease in liver fat. Similar improvements using other diets are discussed.

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Effect of Dietary Protein on the Metabolism of Sodium Acetate-1-C^{14} in Chicks[1]

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Previous studies (Kokatnur et al., '58a, b) indicated that a high protein intake tended to depress the atherogenic effect of dietary cholesterol and fat in chicks. The influence of protein may, in part, have been due to its lipotropic effect as the high serum lipid and cholesterol levels in chicks which had been fed a low-protein diet deficient in methionine and choline was lowered by the simple addition of more protein (Nishida et al., '58). Other workers have also observed that dietary protein influenced serum cholesterol levels (Stamler et al., '58; Johnson et al., '58). Therefore, it appeared that the level of dietary protein must receive as much attention as that of the dietary fat in such studies.

Serum cholesterol levels may be considered representative of a dynamic equilibrium between the rate of introduction and the rate of removal of cholesterol from the blood. As the liver is the main site of both the biosynthesis and degradation of cholesterol, the hypocholesteremic effect of dietary protein could be partially attributed to less biosynthesis of cholesterol or more degradation of cholesterol to bile acids in the liver of birds fed a high- as compared with those fed a low-protein diet.

The present study was designed to observe the effect of dietary protein on the *in vivo* incorporation of acetate-1-C^{14} into liver and serum cholesterol and fatty acids, liver protein and liver glycogen in the presence and absence of dietary cholesterol.

METHODS

One hundred fifty day-old chicks (New Hampshire-Columbian Cross) were fed on a nutritionally-complete diet which contained 35% of soybean protein[2] and 1% of corn oil (Kokatnur et al., '59). After one week 60 chicks of approximately the same weight were selected from the 150 chicks, divided into 4 groups of 15 birds each and fed for three weeks diets which contained 15 or 30% of protein, with and without 1% of dietary cholesterol (table 1). The birds were then injected intraperitoneally with sodium acetate-1-C^{14} (specific activity 1.49 mc/millimole) in physiological saline solution (50 μc/kg of body weight) and bled by heart puncture at 15, 30, 45, 60 and 120 minutes after the injection. The livers were immediately removed, weighed, frozen in liquid nitrogen, wrapped in aluminum foil and stored in screw-cap bottles at −20°C.

TABLE 1
Composition of diets[1]

Constituents	High protein	Low protein
	%	%
Commercial soybean protein[2]	35.30	17.65
Methionine	0.75	0.25
Glycine	0.30	0.15
Choline	0.07	0.07
Corn oil	10.00	10.00
Salt mixture[3]	5.34	5.34
Glucose	48.24	66.54

[1] One per cent of cholesterol was added at expense of glucose to two of the 4 diets. Vitamins were added at the same level as in a previous experimental diet (Nishida et al., '58).

[2] Drackett protein prepared from soybean meal, Drackett Company, Cincinnati; 35.30% or 17.65% of isolated (Drackett) soybean protein is equivalent to 30% or 15% of pure soybean protein, respectively.

[3] Glista salt mixture (Glista, W. A. 1951 The amino acid requirements of the chick: Method and application to some of the amino acids. Doctoral thesis, University of Illinois, Urbana).

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[2] Drackett soybean protein.

The livers were sliced in the frozen state, and weighed samples (approximately 2 gm for the livers from chicks which had been fed cholesterol and 4 gm for those not fed cholesterol) were homogenized with hot absolute ethanol, transferred quantitatively to a 100-ml volumetric flask and diluted to the mark with ether so as to make the final ratio of ethanol to ether approximately 3:1. The stoppered flasks were kept at room temperature for 24 hours with occasional shaking; the extract was filtered into 100-ml volumetric flasks and diluted to volume with ethanol-ether. Aliquots were removed for cholesterol determinations, separation of cholesterol digitonide and fatty acids and determination of total lipids.

The serum sample was obtained as described previously (Nishida et al., '58); 4 ml of serum were extracted with 100 ml of alcohol-acetone (1:1). Aliquots of the extracts were used for cholesterol determination and separation of cholesterol digitonide and fatty acids. Total serum cholesterol was analyzed according to the method of Sperry and Webb ('50), and total liver cholesterol was analyzed as previously described (Nishida et al., '58).

Fifteen milliliters of liver extract or 25 ml of serum extract were used for the quantitative separation of cholesterol digitonide and for counting its radioactivity. Purified digitonide obtained in the same way as for the total serum and liver cholesterol determination was dried in a vacuum oven at 60°C for 24 hours and weighed. The cholesterol digitonide was dissolved in warm methanol and plated on an aluminum planchet, and its radioactivity counted.

For the quantitative separation and determination of radioactivity of the liver or serum fatty acids, 15 ml of the liver or serum extract was transferred into a 100-ml round-bottom flask and the solvent removed under vacuum on a Rinco evaporator. The extract was then saponified with 4 ml of 30% aqueous potassium hydroxide solution-absolute alcohol (1:1) at 80°C for three hours. After saponification, the solution was transferred quantitatively to a cylinder-type separatory funnel and washed 5 times with 20-ml portions of petroleum ether (Skellysolve F) to remove unsaponifiable materials. The remaining solution was transferred to a 100-ml round-bottom flask and the alcohol removed under vacuum on a Rinco evaporator. The aqueous solution was quantitatively transferred to a separatory funnel and acidified to pH 1 with 18 N sulfuric acid. The free fatty acids were removed by three extractions with 20-ml portions of Skellysolve F and the combined extracts washed with two 20-ml portions of 2% acetic acid solutions, and three 20-ml portions of distilled water. The extracts were dried over anhydrous sodium sulfate, filtered, concentrated to a small volume, transferred to a 10-ml volumetric flask with Skellysolve F and diluted to the mark. An aliquot was then mounted on an aluminum planchet, dried, and weighed, and its radioactivity determined. The total lipid content in the liver was determined gravimetrically on the pooled samples of lipid extracts.

The total protein content in the liver was determined by the Kjeldahl method, and the radioactivity of protein was determined on the sample obtained by the following purification of the lipid-free residue. The residue was washed twice with 20-ml portions of alcohol-ether (3:1), once with 20 ml of ether, dried at room temperature under vacuum, washed 4 times with 20-ml portions of warm 5% trichloroacetic acid and twice each with 50% alcohol, absolute alcohol and ethyl ether. The residue was suspended in 20 volumes of Skellysolve F with the aid of an homogenizer, and filtered under light suction through a Tracerlab F8B precipitation apparatus fitted with a filter paper. The filter paper containing the protein layer was dried, removed from the assembly, placed in an aluminum planchet and counted.

The glycogen was separated from a liver sample of approximately 0.5 to 1 gm as described by Hassid and Abraham (Colowick and Kaplan, '57). The crude glycogen was washed twice with 10% aqueous potassium hydroxide solution—alcohol (1:1), with 50% alcohol, and then with 95% ethanol. It was dissolved in a minimal quantity of 5% trichloracetic acid and the remaining protein removed by filtration. The glycogen was reprecipitated by the addition of ethanol,

isolated by centrifugation, washed twice with 50% ethanol, followed by two washings each with 95% ethanol and ether, dried in an oven at 100°C and weighed. For the counting of radioactivity of glycogen, further purification was carried out by reprecipitating glycogen from a minimal amount of water and repeating the above washing procedures. The dried glycogen thus obtained was suspended in absolute ethanol and its specific activity was determined in the same way as for liver protein.

The radioactivity on each planchet was counted with a Packard Instrument Gas Flow Counter, corrected for mass absorption, and the specific activity obtained. Before counting material on the planchet, it was dried under an infrared lamp and weighed on a semi-microbalance. The results for liver constituents were expressed as percentage incorporation of injected sodium acetate-1-C^{14} into each material. For serum cholesterol and fatty acids, the results were expressed as counts per minute of each material, per 100 ml of serum.

RESULTS

A higher initial incorporation of acetate-1-C^{14} into liver cholesterol occurred in chicks which had continued to be fed a low-protein diet than into the liver cholesterol of those fed a high-protein diet (fig. 1). Furthermore, the presence of dietary cholesterol significantly depressed the percentage of incorporation of acetate-1-C^{14} into liver cholesterol. In the present study the amount of liver constituents in each group of chicks varied with the dietary protein level and with

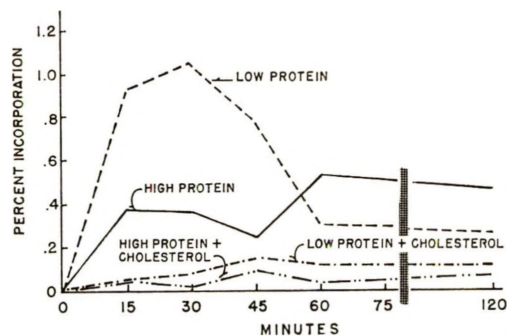

Fig. 1 Incorporation of acetate-1-C^{14} into liver cholesterol.

TABLE 2

Analysis of serum cholesterol and liver constituents

Diet	Body weight	Liver weight	Total serum cholesterol	Total liver cholesterol	Total liver lipid	Total liver fatty acids	Liver protein	Liver glycogen
	gm	*gm*	*mg/100 ml*	*mg/100 ml*	%	%	%	%
High protein	428 ± 1[1]	11.2 ± 0.3	121 ± 5	289 ± 7	5.91	3.18 ± 0.11	21.08 ± 0.33	9.95 ± 0.88
Low protein	300 ± 5	8.0 ± 0.4	161 ± 7	289 ± 3	7.17	3.66 ± 0.13	19.01 ± 0.37	11.53 ± 1.07
High protein + cholesterol	399 ± 2	11.0 ± 0.3	178 ± 12	1028 ± 215	7.15	3.26 ± 0.15	19.87 ± 0.30	8.63 ± 0.57
Low protein + cholesterol	267 ± 3	9.1 ± 0.3	395 ± 42	2153 ± 197	10.03	4.26 ± 0.30	16.24 ± 0.45	10.59 ± 1.13

[1] Standard error of mean.

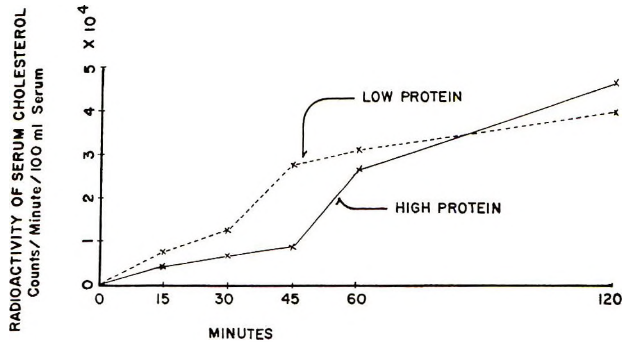

Fig. 2 Incorporation of acetate-1-C^{14} into serum cholesterol in the absence of dietary cholesterol.

the presence of dietary cholesterol. Therefore, it seemed best to compare the percentage of incorporation of injected C^{14} into each liver constituent. Although many workers use the specific activity to compare incorporation rate in an *in vivo* experiment, specific activity should be used only for comparison of radioactive compounds which are subject to the same degree of dilution effect.

The liver cholesterol concentration of birds consuming diets containing 30 or 15% of protein increased 3.5- or 7-fold, respectively, when 1% of cholesterol was added to the diets (table 2). These results indicated that the elevating effect of dietary cholesterol on the liver cholesterol level was more pronounced at the low-protein than at the high-protein level. However, in the presence of cholesterol, the percentage of incorporation of acetate-1-C^{14} into liver cholesterol in chicks which had continued to be fed a low-protein diet was greater than in those receiving a high-protein diet. In the absence of dietary cholesterol, the liver cholesterol level of birds in both groups did not differ. Yet the birds in the low-protein group incorporated a significantly higher initial percentage of acetate-1-C^{14} into liver cholesterol. Maximum incorporation of acetate into liver cholesterol occurred 45 minutes after the injection of acetate-1-C^{14} into birds fed cholesterol-containing diets, and 30 minutes for those which continued to be fed the low-protein diet containing no dietary cholesterol. Birds kept on a cholesterol-free high-protein regimen did not show a clear maximum incorporation time.

The initial percentage of incorporation of acetate-1-C^{14} into serum cholesterol was also higher in birds fed the low-protein diet than in those receiving the high-protein diet in spite of a significantly higher serum cholesterol level in the former (fig. 2). However, at both dietary protein levels, maximum incorporation was not reached until two hours or more after the injection of acetate. In the presence of dietary cholesterol, only negligible incorporation into serum cholesterol was observed at both high- and low-dietary protein levels.

A low-protein diet significantly elevated the total liver fatty acid content and also increased the incorporation of acetate-1-C^{14} into liver fatty acids (fig. 3). Although dietary cholesterol depressed cholesterol biosynthesis, it stimulated the incorporation of acetate-1-C^{14} into fatty acids. Maximum incorporation occurred 45 minutes

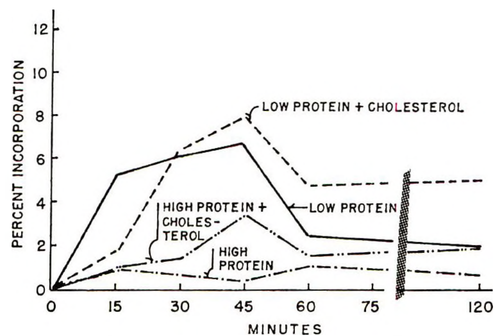

Fig. 3 Incorporation of acetate-1-C^{14} into liver fatty acids.

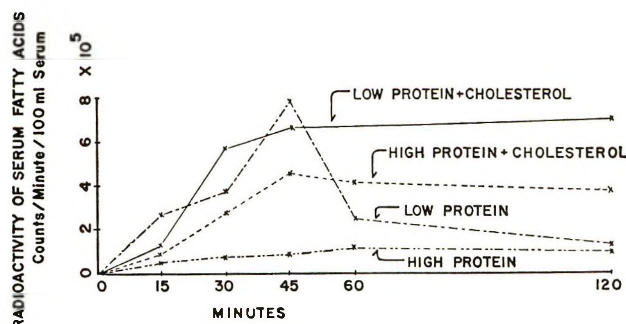

Fig. 4 Incorporation of acetate-1-C^{14} into serum fatty acids.

after injection of acetate-1-C^{14}. Similar results were obtained with the incorporation of injected acetate-1-C^{14} into serum fatty acids (fig. 4).

The relationship between fatty acid biosynthesis and cholesterol biosynthesis was compared by plotting the ratio of incorporation of acetate into fatty acids to its

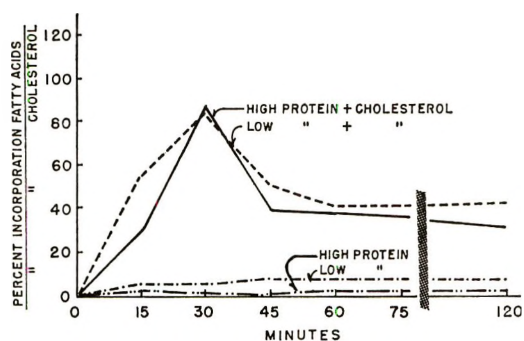

Fig. 5 The ratio of acetate-1-C^{14} incorporation into liver fatty acids to the incorporation into liver cholesterol.

incorporation into cholesterol. In the absence of dietary cholesterol, the low-protein diet significantly increased the incorporation ratio (fig. 5), indicating that the elevating effect of the low-protein diet on the rate of fatty acid biosynthesis was more pronounced than on the rate of cholesterol biosynthesis. In the presence of dietary cholesterol, no significant difference in incorporation ratios was observed between chicks kept on low- and high-protein regimens. However, the incorporation ratios were significantly increased because of the elevating effect of dietary cholesterol on fatty acid biosynthesis and its depressing effect on cholesterol biosynthesis. It appeared that when cholesterol was fed in the diet, the liver synthesized more fatty acids in order to esterify more exogeneous cholesterol for absorption and transportation. The effect of dietary protein on the ratio of acetate-1-C^{14} incorporation into serum fatty acids

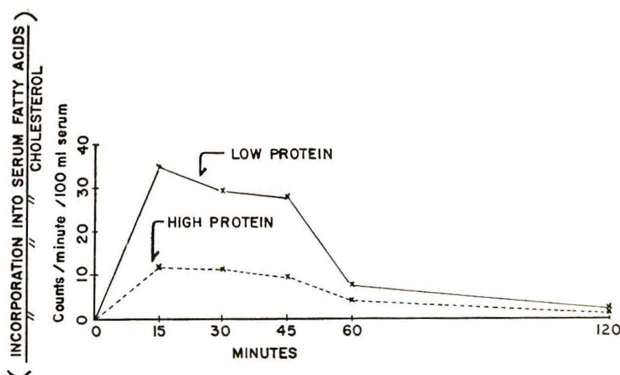

Fig. 6 The ratio of acetate-1-C^{14} incorporation into serum fatty acids to the incorporation into serum cholesterol in the absence of dietary cholesterol.

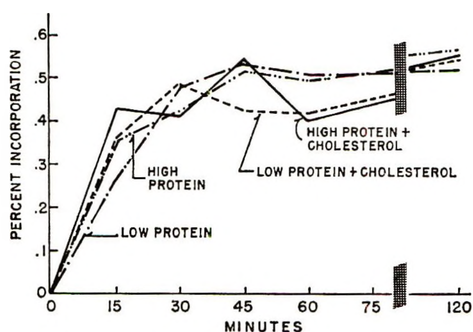

Fig. 7 Incorporation of acetate-1-C^{14} into liver protein.

to the incorporation into serum cholesterol was similar to the ratio of incorporation into liver fatty acids and liver cholesterol (fig. 6).

The liver protein level was significantly higher in birds fed high-protein diets than in those fed low-protein diets. Although a low-protein diet and the presence of dietary cholesterol significantly lowered the liver protein level, the percentage of incorporation of acetate-1-C^{14} into liver protein was not affected by these dietary factors (fig. 7). A higher liver lipid and a lower liver protein content resulted in chicks kept on the low-protein regimen with cholesterol as compared with those fed the high-protein diet with cholesterol (table 2).

No significant effect of dietary protein and cholesterol on the glycogen content in the liver was noted. The percentage of incorporation of acetate into liver glycogen remained low, from 0.002 to 1.02%, and was also not affected by the dietary protein level or by the presence of dietary cholesterol.

DISCUSSION

A marked inhibition of the *in vivo* and *in vitro* biosynthesis of cholesterol has been reported in several species of animals fed dietary cholesterol[3,4] (Gould et al., '53; Tomkins et al., '53a; Langdon and Bloch, '53). Furthermore, the inhibition of cholesterol biosynthesis was attributed to a high liver cholesterol level (Alfin-Slater et al., '52; Franz et al., '54). There is general agreement that the liver cholesterol level is under homeostatic regulation by dietary cholesterol (Bucher et al., '59) and that cholesterol precursors, such as squalene, lathosterol, and 7-dehydrocholesterol inhibit cholesterol biosynthesis (Langdon and Bloch, '53; Tomkins et al., '53b). Furthermore, Mukherjee and Alfin-Slater ('58) concluded that the accumulation in the liver of endogenous cholesterol as well as exogenous cholesterol depressed cholesterol synthesis by this organ.

In the present study, cholesterol biosynthesis was depressed in chicks fed 1% of cholesterol, but the effect of a low-protein intake in stimulating cholesterol biosynthesis could be demonstrated even when cholesterol concentration in the liver was high. However, under these conditions the stimulation was very much less than that observed when liver cholesterol was low. It would therefore appear that cholesterol biosynthesis in the liver is influenced by factors other than liver cholesterol concentration.

The present results also appeared to indicate a close relationship between cholesterol biosynthesis and fatty acid biosynthesis. An increase in cholesterol biosynthesis was accompanied by an increase in fatty acid biosynthesis in chicks fed a low-protein diet as compared with those fed a high-protein diet. Furthermore, a dietary source of cholesterol appeared to stimulate fatty acid biosynthesis. It has been reported that dietary cholesterol profoundly affected the biosynthesis of cholesterol, but that there was no diminution in fatty acid synthesis (Gould et al., '53). However, this conclusion was attained from experiments using liver slices and does not necessarily represent *in vivo* fatty acid synthesis which exists in a dynamic state. From our present studies it may be stated that, when the liver has a tendency to accumulate endogenous or exogenous sources of cholesterol, it can synthesize more fatty acids. These fatty acids may partially serve for the transport of endogenous or exogenous sources of liver cholesterol or for the absorption of dietary cholesterol as cholesterol esters.

[3] Gould, R. G., and C. B. Taylor 1950 Effect of dietary cholesterol on hepatic cholesterol synthesis. Federation Proc., *9:* 179 (abstract).

[4] Gould, R. G., and G. Popjak 1957 Biosynthesis of cholesterol *in vivo* and *in vitro* from DL- β-hydroxy- β-methyl- δ-(2-C^{14})-valerolactone. Biochem. J., *66:* 51P (abstract).

We have previously stated that an important relationship existed between the energy to protein or E/P ratio and the serum and carcass cholesterol level in chicks (Kokatnur et al., '58b). A higher E/P ratio or low-protein level was found to increase serum cholesterol values, regardless of the type of fat in the diet. In the present studies, it was noted that a higher E/P ratio accelerates a general lipogenesis in chicks. On the other hand, fasting decreased not only hepatic cholesterol biosynthesis but also fatty acid biosynthesis, which seemed to indicate that caloric restriction inhibits general lipogenesis (Lyon et al., '52; Hutchens et al., '54). Therefore, lipogenesis may be affected by caloric balance as well as impairment of nutrition.

We have also previously reported that the imbalance of dietary cholesterol, fat, and methionine at the low-protein level was particularly atherogenic in chicks (Nishida et al., '58). In the present study, the level of choline was below the optimum need for chicks although the total liver lipid content in the chicks did not indicate a signficant deficiency in lipotropic factors. The beneficial effect of a high-protein diet on cholesterol metabolism cannot be explained fully by the presence of an optimum amount of methionine or by the lipotropic action of excess protein as a precursor of choline. The elevating effect of a low-protein diet on the serum cholesterol level in chicks may be due to a combination of factors such as shortage of lipotropic materials, pronounced lipogenesis, and possibly cholesterol catabolism (Nishida et al., '60).

SUMMARY

A low dietary-protein level tended to increase at least initial incorporation of acetate-1-C^{14} into liver cholesterol in chicks. In the presence of dietary cholesterol, the percentage of incorporation of acetate-1-C^{14} into liver cholesterol in birds which had been kept on a low-protein dietetary regimen was greater than in those fed a high-protein diet. However, dietary cholesterol depressed significantly the rate of incorporation of acetate into liver cholesterol. The elevating effect of a low-protein diet on the biosynthesis of cholesterol appeared to be independent of the liver cholesterol level. A low-protein diet also increased the rate of fatty acid biosynthesis, and its elevating effect on fatty acid biosynthesis was more pronounced than on cholesterol biosynthesis. Although dietary cholesterol depressed cholesterol biosynthesis, it stimulated fatty acid biosynthesis in chicks. The effect of dietary protein and cholesterol on the incorporation of acetate into serum cholesterol and serum fatty acids was similar to that in liver cholesterol and liver fatty acids. The liver protein level was significantly reduced in birds fed a low protein diet or dietary cholesterol, yet the incorporation of acetate into liver protein was not affected by these dietary factors. No significant effect of dietary protein or cholesterol was observed on the incorporation of acetate into liver glycogen.

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Chemical Pathology of Nutritional Deficiency Induced by Certain Plant Proteins

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Earlier studies (Sidransky and Farber, '58a,b, Sidransky and Baba, '60) have been published on the pathologic lesions produced in young rats force-fed for three to 7 days with purified diets devoid of certain single essential amino acids. These lesions resembled many of the alterations described in kwashiorkor, a disease of malnourished infants (Trowell et al., '54). The results of these experiments raised the question whether similar changes might be induced by force-feeding diets in which the protein was supplied by some of the natural plant foodstuffs, known to be deficient in certain amino acids. Therefore, the foodstuffs selected for the present study were those largely consumed in regions where kwashiorkor is found (Trowell et al., '54). Among these are corn, rice, wheat, cassava and milo. Therefore diets composed of each of these 5 foodstuffs as the sole source of protein, with supplements of other essential nutrients, were force-fed to young rats for three days or fed ad libitum for 7 days. Morphologic changes in organs of the rats thus fed were studied, and selected biochemical determinations on these organs were also made. The findings, both morphologic and biochemical, closely resembled those previously noted in acute essential amino acid deficiencies[1] (Adamstone and Spector, '50; Spector and Adamstone, '50; Van Pilsum et al., '57; Sidransky and Farber, '58a,b; Sidransky and Baba, '60), as well as many of the pathologic findings observed in kwashiorkor.

METHODS

Male and female rats of the Sprague-Dawley strain, one-month old and weighing an average of 75 gm, were obtained from the breeding colony at the National Institutes of Health. They were maintained on laboratory chow[2] for a few days and then fasted overnight before being fed the special diets. All animals had access to water. In every experiment from three to 6 groups of rats of approximately the same sex, age and weight were used. They were housed in individual wire cages with raised bottoms in an air-conditioned room maintained at 25.6°C.

The experimental diets were similar to those described by Shils and co-workers, ('54). Table 1 shows the composition and protein content of each. The vitamin-sucrose mixture contributed the following number of milligrams of vitamins to each 100 gm of diet: thiamine·HCl, 0.25; riboflavin, 0.5; pyridoxine·HCl, 0.25; Ca pantothenate, 2.0; nicotinic acid, 1.0; choline chloride, 100.0; biotin, 0.01; folic acid, 0.1; inositol, 10.0; 2-methyl-1, 4-naphthoquinone, 0.1; cyanocobalamin (vitamin B_{12}), 0.01; and cod liver oil, 0.1. The salt mixture was that described by Hegsted et al., ('41). Each ration was blended with water so that one milliliter of mixture contained 0.67 gm of diet. The resulting mixture could be administered by stomach tube.

Force-feeding was performed with plastic tubes according to the method of Shay and Gruenstein ('46). The ration was given daily at 9 A.M., 3 P.M. and 9 P.M. for three days. The rats received 6 gm of diet the first day, 8 gm the second and 10 gm the third. The average daily intake per rat was 1.1 gm of diet per 10 gm of

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[1] Samuels, L. T., H. C. Goldthorpe and T. F. Dougherty 1951 Metabolic effects of specific amino acid deficiencies. Federation Proc., *10*: 393 (abstract).

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

TABLE 1
Composition of diet

	Experimental foodstuff	Diet	Protein in diet[1]
		%	%
Diet 1	Cornmeal, white[2]	(74)	6.1
Diet 2	Rice flour[3]	(74)	6.1
Diet 3	Wheat flour, white[4] Sucrose	(55.7) (18.3)	8.2
Diet 4	Milo (grain sorghum)[5]	(74)	9.0
Diet 5	Cassava flour[6]	(74)	1.1
	Added to all diets		
	Corn oil[7]	17	
	Vitamin-sucrose mixture[8]	5	
	Salt mixture[8]	4	
	Total	100	

[1] Nitrogen determined by Kjeldahl.
[2] Indian Head Cornmeal, bolted, water-ground, Wilkins-Rogers Milling Company, Washington, D. C.
[3] River Brand Rice Flour, Memphis, Tennessee.
[4] Seal of Colorado Flour, bleached, unenriched, Colorado Mill and Elevator Company, Denver, Colorado.
[5] Finely ground milo (grain sorghum) (Kafir 44–14) obtained from Dr. J. C. Hillier, Animal Husbandry Department, Oklahoma State University, Stillwater, Oklahoma.
[6] Tapioca flour, Stein Hall and Company, Inc., New York.
[7] Mazola, Corn Products Refining Company, New York.
[8] See text.

initial body weight. On the morning of the 4th day the rats were anesthetized with ether and exsanguinated, approximately 14 hours after the last feeding. Control animals of the same size and sex had received stock laboratory chow[3] ad libitum and had been fasted for 14 hours before being killed.

The groups of rats in the ad libitum experiments were equivalent to those in the force-feeding experiments. These rats were fasted overnight before starting to receive the special diets for 4 days. Since few alterations were found by then, the diet was continued for 7 days in one experiment in the hope of obtaining a better comparison with the group force-fed for three days. The diet mixture prepared without added water was available to the rats in the ad libitum groups at all times. Finely ground stock diet was available to the control group.

Rats were weighed at the beginning and end of each experiment. The organs were weighed fresh, using the right organ, only, of paired organs.

Collection of material. Pieces from selected organs were fixed in Zenker-formol solution and in 10% formalin. Paraffin sections were prepared and routinely stained with hematoxylin and eosin. Some selected sections were also stained with Best's carmine and by the periodic acid-Schiff method. Frozen sections of liver were stained with Oil red O.

Pieces of liver from the median and left lateral lobes were rapidly removed, weighed and placed in 30% KOH for glycogen determination. Another weighed piece was homogenized in distilled water and a suitable aliquot added to an equal volume of 10% trichloroacetic acid (TCA) for protein determination. One large piece of liver was weighed and then frozen at −15°C for subsequent lipid determination.

Chemical analyses. Protein was measured by determination of Kjeldahl nitrogen (Perrin, '53) on TCA precipitates of liver aliquots. The aliquots were washed in succession with 5% TCA, 95% ethanol, ethanol-ethyl ether mixture (3:1) and ethyl ether before digestion.

For total lipid, the frozen aliquot of liver was thawed, ground to a dry powder with anhydrous sodium sulfate and extracted with chloroform for 24 hours. After evap-

[3] Purina Laboratory Chow.

oration of the chloroform, the residue was extracted with petroleum ether and the lipid remaining after evaporation of this solvent was weighed. Aliquots of this lipid residue dissolved in chloroform were used for determinations of cholesterol and phospholipid. Cholesterol was determined by the method of Carr and Drekter ('56), and phospholopid by digesting and measuring P by the method of Fiske and Subbarrow ('25).

Liver glycogen was determined on approximately 1-gm aliquots of liver by the method of Seifter et al., ('50).

RESULTS

Force-feeding experiments

The body and organ weights of each group of animals in the force-feeding experiments are summarized in table 2. The force-fed animals of the experimental groups are compared with control animals fed the stock diet ad libitum. These animals fed the stock diet had the same average body weight at sacrifice as the experimental animals. An additional control group was composed of rats force-fed a complete ration containing purified amino acids as the sole source of nitrogen for three days. These force-fed control animals were fed a diet mixture described previously (Sidransky and Baba, '60).

The rats force-fed the corn diet gained the most weight, 7.1 gm in three days, whereas the animals fed the wheat, rice or milo diets gained less. The animals force-fed the cassava diet lost weight, 0.8 gm in three days. The livers of the animals fed the corn, rice, wheat or milo diets were heavier than those of rats fed the cassava or stock diets. Examination of the other organs revealed that the submaxillary gland and the spleen were frequently significantly smaller in most of the rats force-fed the experimental diets than in rats fed the stock diets. The kidney and gastrocnemius muscle in some experimental groups were significantly smaller than those in the stock-fed group.

In table 3 are shown the amount of lipid, glycogen and protein in the livers of animals force-fed. There is a significant increase of liver lipid and glycogen in comparison with those fed the stock control diet. The increase in liver lipid is largely in neutral fat. A significant decrease of liver protein was observed in all groups except in that fed the milo, in comparison with the group fed the stock diet. The extremely low level of liver glycogen in the stock control animals may have resulted because no food was taken for 14 hours before killing, whereas the experimental animals were force-fed 3.4 gm of diet 14 hours before killing. In one experiment, control rats fed the stock diet were tube-fed a sucrose solution isocaloric with the 3.4 gm of experimental diet 14 hours before killing. The average liver glycogen in these animals was 93 mg per liver. The results for body and organ weights and liver composition in animals force-fed the complete purified diet (tables 2 and 3) are very similar to those obtained for the control stock diet-fed animals and indicate that the force-feeding procedure itself is not responsible for any major differences.

In one experiment a group of animals was fore-fed the corn diet supplemented with 1.0% of L-lysine and 0.5% of L-tryptophan. In these animals changes in the liver and other organs were identical with changes found in animals fed the unsupplemented corn diet but over-all growth was somewhat better.

In one experiment the lipid intake was decreased and the effect of this on animals receiving the plant foodstuff diets was investigated. Groups of rats were force-fed rice and cassava diets containing 5% instead of 17% of corn oil. Sucrose was substituted for the difference in corn oil. Animals receiving these modified diets showed the same changes as those fed the regular rice and cassava experimental diets.

Morphologic changes. Liver. Observations on the liver of the control animals after routine hematoxylin and eosin staining failed to show any vacuolation indicative of an abnormal amount of fat or glycogen. After using the special stains, Oil red O and Best's carmine, little fat and glycogen were detected (figs. 1 and 3). In rats force-fed each of the 5 experimental diets the liver had a glistening yellowish-brown appearance. Histologically, after hematoxylin and eosin staining the cytoplasm of the hepatic cells was vacuo-

TABLE 2

Body and organ weights of rats fed corn, rice, wheat, cassava or milo diets[1]

Diet	Rats	Body weight			Liver	Pancreas	Submaxillary gland	Kidney	Gastrocnemius muscle	Spleen	Testis
		Initial	Final	Change							
	No.	*gm*	*gm*	*gm*	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Three-day force-feeding experiments											
Corn	20	67.1	74.0	+7.1	3.43 ± 0.09[2,3]	330 ± 11[3]	64 ± 3.1[3]	331 ± 6[3]	350 ± 11[3]	231 ± 28[3]	435 ± 24
Rice	19	70.5	76.6	+6.2	3.47 ± 0.09[3]	379 ± 8	68 ± 2.9[4]	344 ± 7[4]	392 ± 8	308 ± 33	457 ± 22
Wheat	16	73.1	80.0	+6.9	3.33 ± 0.14	415 ± 16	69 ± 2.0[4]	332 ± 9	376 ± 11	247 ± 12[3]	467 ± 24
Cassava	15	74.4	73.6	−0.8	2.98 ± 0.13	381 ± 15	47 ± 2.7[3]	346 ± 10	346 ± 15[3]	185 ± 19[3]	410 ± 21
Milo	9	75.2	77.5	+2.3	3.27 ± 0.17	398 ± 14	75 ± 2.3	345 ± 8	385 ± 11	269 ± 16[3]	492 ± 21
Stock	22	—	77.5	—	2.98 ± 0.11	381 ± 12	78 ± 2.9	372 ± 9	401 ± 13	373 ± 29	403 ± 32
Complete amino acid	14	71.2	76.7	+5.5	3.04 ± 0.14	365 ± 9	72 ± 3.9	368 ± 7	376 ± 11	241 ± 15	478 ± 20
Seven-day ad libitum experiment											
Corn	5	62.0	66.6	+4.6	3.11 ± 0.05[3]	324 ± 17[4]	63 ± 3.0[3]	298 ± 3[3]	270 ± 17[3]	229 ± 30[4]	436 ± 41
Rice	5	64.7	70.6	+5.9	3.07 ± 0.12[3]	348 ± 13	74 ± 8.8	298 ± 8[3]	335 ± 21[4]	240 ± 13[4]	489 ± 22
Wheat	5	61.8	64.2	+2.4	2.94 ± 0.10[3]	346 ± 28	57 ± 6.9[3]	282 ± 6[3]	315 ± 11[3]	248 ± 15[4]	519 ± 37
Cassava	5	63.3	56.8	−6.5	2.52 ± 0.05[3]	281 ± 17[4]	52 ± 4.9[3]	274 ± 13[3]	247 ± 20[3]	176 ± 16[3]	376 ± 30
Milo	5	61.3	63.4	+2.1	2.82 ± 0.16[3]	302 ± 23	57 ± 1.1[3]	304 ± 11[3]	295 ± 22[3]	243 ± 44[4]	476 ± 56
Stock	3	63.8	90.2	+26.3	4.02 ± 0.07	511 ± 72	99 ± 6.6	455 ± 32	400 ± 3	710 ± 127	551 ± 67

[1] Observations of rats fed experimental diets compared with those fed stock diets ad libitum.

[2] Mean value ± standard error of the mean.

[3] $P < 0.01$ (highly significant).

[4] P between 0.01 and 0.05 (probably significant).

TABLE 3

Liver lipid, glycogen and protein of rats fed corn, rice, wheat, cassava or milo diets[1,2]

Diet	Liver lipid				Liver glycogen	Liver protein
	Total lipid	Cholesterol	Phospholipid	Neutral fat[3] (by difference)		
	mg/liver	*mg/liver*	*mg/liver*	*mg/liver*	*mg/liver*	*mg/liver*
Three-day force-feeding experiments						
Corn	(19) 259 ±22[4]	(5) 14.4 ±1.9	(5) 39.7 ±4.4	(5) 182 ±10[4]	(9) 188 ±37[4]	(5) 451 ±10[4]
Rice	(18) 210 ±14[4]	(8) 12.6 ±1.2	(9) 49.3 ±7.0	(8) 131 ±18[4]	(14) 164 ±27[4]	(9) 498 ±17[5]
Wheat	(16) 200 ±15[4]	(11) 11.9 ±0.6	(11) 60.2 ±4.4	(11) 140 ±21[4]	(15) 157 ±25[4]	(14) 500 ±15[5]
Cassava	(15) 208 ±13[4]	(12) 11.2 ±1.0	(12) 54.7 ±4.4	(12) 152 ±15[4]	(15) 103 ±16[4]	(13) 414 ±16[4]
Milo	(8) 195 ±17[4]	(8) 13.8 ±0.8	(8) 64.1 ±4.4	(8) 117 ±16[5]	(9) 124 ±24[4]	(8) 566 ±26
Stock	(18) 130 ±6	(13) 11.0 ±0.7	(14) 49.3 ±6.6	(13) 76 ±6	(18) 3 ±1	(11) 572 ±23
Complete amino acid	(14) 141 ±5				(14) 64 ±16	(14) 530 ±19
Seven-day ad libitum experiment						
Corn	(5) 113 ±5[4]				(5) 317 ±19	(5) 372 ±10[4]
Rice	(5) 164 ±18				(5) 337 ±35	(5) 384 ±20[4]
Wheat	(5) 153 ±20				(5) 323 ±31	(5) 363 ±10[4]
Cassava	(5) 123 ±4[4]				(5) 247 ±41	(4) 308 ±17[4]
Milo	(5) 171 ±32				(5) 250 ±27	(5) 379 ±10[4]
Stock	(3) 172 ±11				(3) 141 ±50	(5) 768 ±25

[1] Observations of rats fed experimental diets compared with those fed stock diets ad libitum.
[2] Numbers in parentheses indicate number of animals in group followed by the mean value ± standard error of the mean.
[3] Neutral fat = total liver lipid − (cholesterol plus phospholipid). See text for explanation.
[4] $P < 0.01$ (highly significant).
[5] P between 0.01 and 0.05 (probably significant).

lated predominantly in the periportal areas (fig. 2). With special stains, Oil red O and Best's carmine, the liver cells showed considerable amounts of both lipid and glycogen. The lipid was confined to the hepatic cells about the portal triads (fig. 4). The glycogen was diffusely distributed. The livers from the various groups were graded according to the amount of lipid observed histologically. The order, from most to least lipid in the livers, was corn, rice, cassava, wheat, and milo.

Pancreas. The pancreas was normal in the control animals (fig. 5). In rats fed the corn diet the acinar cells of the pancreas showed mild-to-moderate decrease in the cytoplasmic/nuclear ratio and the number of zymogen granules was decreased in comparison with controls. The nuclei were somewhat crowded together and appeared to be disorderly in arrangement (fig. 6). The islets were normal. With the corn diet such changes were found in the majority of the animals but not in all. Animals fed the rice, wheat and cassava diets revealed simliar atrophic pancreatic changes in approximately half of the animals. Animals receiving the milo diet showed no changes.

Submaxillary gland. In rats fed the corn, rice, cassava and wheat diets, the submaxillary glands were usually smaller and histologically the acinar cells revealed a marked loss of cytoplasm in comparison with the glands of control animals (figs. 7 and 8).

Spleen. The spleen in the control animals was normal. That of animals fed all of the experimental diets showed atrophic changes consisting of a reduction in lymphocytes and prominence of the connective tissue.

The following organs showed no gross or microscopic changes: heart, lung, kidney, adrenal, stomach, small intestines and muscle.

Ad libitum experiments

The body and organ weights of animals on the different diets in a 7-day ad libitum experiment are summarized in table 2. While control animals fed the stock diet ate well and gained weight, those receiving the experimental diets ate less and gained less weight. Rats fed the stock diet ate the largest amount of food, 9 gm per rat a day, whereas those receiving the experimental diets ate the following amounts in grams per rat per day: corn, 5.3; rice, 6.1; wheat, 5.5; cassava, 4.9; and milo, 5.8. Of the experimental animals, those fed the rice diet gained the most weight, whereas those receiving the cassava diet lost weight. In comparison with the rats fed the stock diet, the weights of liver, submaxillary gland, kidney, gastrocnemius muscle and spleen were usually significantly less in the experimental groups. The chemical determinations (table 3) showed that the liver protein in all experimental groups and the liver lipid in some groups were decreased in comparison with those of animals fed the stock diet. On the other hand, the liver glycogen in the experimental animals showed a higher value than that in the rats receiving the stock diet.

Morphologic changes. In the 7-day ad libitum-fed experimental animals the only morphologic changes observed were in the liver. The liver of occasional animals in each of the experimental groups showed mild periportal lipid accumulation.

Influence of quantity of diet consumption on pathologic changes

Since the quantity of intake of experimental diets appeared to influence the morphologic and chemical results, several additional force-feeding and ad libitum experiments with the corn and rice diets were carried out. The results are summarized in part in table 4, along with data from tables 2 and 3 for comparison. The greater the amount of corn or rice diet consumed, either by force-feeding or ad libitum feeding, the greater the daily weight gain; and with this gain, the liver weight increased correspondingly due largely to increased liver lipid. When the corn diet was fed at a low level, 4.3 gm per rat a day, the changes in body weight, liver weight and liver lipid were similar in the animals force-fed or fed ad libitum.

Morphologically, pathologic changes similar to those described earlier were found predominantly in the liver, pancreas and submaxillary gland of the animals force-fed the greatest quantity of corn or rice diet. In addition, detailed review of

TABLE 4

Body weight, and liver weight and composition of rats as influenced by corn or rice diets

Diet	Rats	Diet consumed	Types of feeding	Duration	Weight change	Liver			
						Weight	Lipid	Glycogen	Protein
	no.	*gm/day*		*days*	*gm/day*	*gm*	*mg*	*mg*	*mg*
Corn	12	4.3	Force-feeding	4	+0.3	2.07 ± 0.06	65 ± 11	—	—
	8	4.3	Ad libitum	4	+0.6	2.20 ± 0.13	99 ± 8	—	—
	5	5.4	Ad libitum	7	+0.7	3.11 ± 0.05	113 ± 5	317 ± 19	370 ± 10
	20	8.0	Force feeding	3	+2.4	3.43 ± 0.09	259 ± 22	188 ± 37	451 ± 10
Rice	5	5.0	Ad libitum	4	−0.3	2.14 ± 0.10	115 ± 5	—	—
	5	6.1	Al libitum	7	+0.8	3.07 ± 0.12	164 ± 18	337 ± 35	384 ± 20
	19	8.0	Force-feeding	3	+2.1	3.47 ± 0.09	210 ± 14	164 ± 27	498 ± 17

data of the 7-day ad libitum feeding experiment described earlier (table 3) revealed that individual rats of each group eating the largest quantity showed some pathologic changes. On the other hand, rats eating very little showed minimal, if any, pathologic changes.

DISCUSSION

The observations in this study show that pathologic changes in the liver, spleen, pancreas and submaxillary gland can be induced within three days in young rats when the animals are force-fed certain plant foodstuffs as the sole source of protein. The changes are most striking in animals force-fed a corn diet.

On the other hand, when animals were fed the identical plant food stuff diets ad libitum for 7 days, fewer and less severe pathologic changes were noted. On chemical analyses of livers from rats force-fed and from those offered the same diets ad libitum, the force-fed animals showed an increase in liver weight, lipid and glycogen with a decrease in liver protein when compared with the livers of animals fed the stock diet ad libitum. In contrast, the rats fed the same experimental plant foodstuff diets ad libitum had smaller livers, with a smaller amount of lipid and protein than the livers of control animals fed the stock diet for 7 days. These differences can be related to the quantity of the deficient diet consumed. The animals force-fed received the same large amounts by weight of the experimental diets in all groups, whereas with ad libitum feedings the majority of the animals in the experimental groups consumed relatively little.

The pathologic changes found in force-feeding deficiency experiments are different from those obtained with ad libitum feeding. The differences observed between the two types of feeding with amino acid deficiencies have been discussed previously (Sidransky and Farber, '58a,b). In general, with ad libitum feeding, animals fed the deficient diet eat little and lose much body and organ weight. These experimental animals, especially in a critical experiment, develop few histopathologic changes. Such changes induced by a deficient diet are therefore dependent on the quantity of diet consumed.

The most consistent observation in our present experiments was fatty change of the liver. The fatty liver of these rats had a periportal distribution of lipid. A similar alteration in the liver has been described in rats in other experiments, particularly in force-feeding experiments, using diets deficient in certain essential amino acids. Among these are: tryptophan (Adamstone and Spector, '50; Samuels et al., '51; Van Pilsum et al., '57); methionine (Sidransky and Farber, '58a,c); phenylalanine (Samuels et al., '51; Van Pilsum et al., '57); threonine (Sidransky and Farber, '58a,c); histidine (Sidransky and Farber, '58a); valine (Sidransky and Baba, '60); and lysine (Sidransky and Baba, '60). This fatty change in the liver also appeared in ad libitum feeding with certain essential amino acid deficiencies (Cole and Scott, '54; Pomeranze et al., '59). A fatty liver with a periportal distribution in animals fed ad libitum diets deficient in protein was described by Wang and co-workers, ('49), Jaffe et al., ('49), Shils et al., ('54), Best and associates, ('55), and Lucas and Ridout ('55). In these last studies, weeks to months were required to induce the liver alteration. In the force-feeding experiments reported here, this change was induced within three days.

Our own studies and those discussed above indicate many similarities between the findings in studies of rats force-fed diets either devoid of certain single essential amino acids or containing proteins derived from certain plant foodstuffs and the clinical and pathologic observations of human cases of kwashiorkor. Kwashiorkor is generally considered to be the result of protein malnutrition, and often follows an intake of inadequate and poor-quality proteins (Trowell et al., '54). Along with the deficient protein intake the caloric intake is usually adequate or even high (Waterlow, '48; Housden, '50; Davies, '52; Brock and Autret, '52; Brock and Hansen, '58). Often when kwashiorkor develops, the mother has fed the recently weaned infant or young child as much food as possible, and such forcible feeding of infants with an excess of carbohydrates has been reported from many areas (Linden, '54; Platt, '58). The conditions in the human infant are thus similar to the experimental conditions in the force-fed rats. It is conceivable that the nutritional deficiency or imbalance induced in the rat by force-feeding may be closely related etiologically to that occurring in the infant with kwashiorkor.

SUMMARY

Young rats force-fed certain plant foodstuffs as the sole source of protein and supplemented with other essential nutrients developed a periportal fatty liver, excess hepatic glycogen and atrophy of the spleen, pancreas and submaxillary gland within three days. These changes were most marked with corn, rice, and cassava diets and less marked with wheat and milo diets. Animals fed the same diets ad libitum for 7 days, in contrast with those force-fed, consumed less food and showed fewer pathologic changes. The difference in results between the force-feeding and the ad libitum feeding are thus related to the quantity of the diets consumed. The pathologic lesions found in the rats force-fed certain plant foodstuffs resembled some of the alterations found with kwashiorkor, a disease of malnourished infants.

ACKNOWLEDGMENTS

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PLATE 1

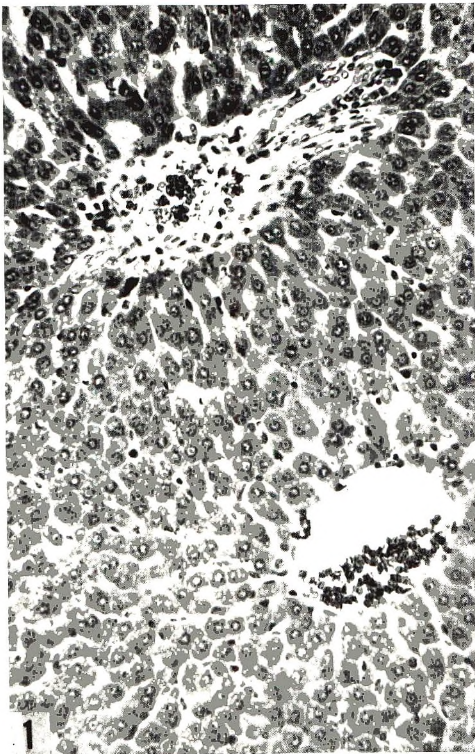
EXPLANATION OF FIGURES

1 Liver of control stock diet fed rat showing a periportal area on left and area of central vein on right. Hematoxylin and eosin. × 200.

2 Liver of rat force-fed the cassava diet. Vacuolation of hepatic cells is prominent in periportal area (left) with only few vacuoles in central area (right). Hematoxylin and eosin. × 200.

3 Liver of control stock-fed rat. Note absence of fat. Oil red O stain. × 66.

4 Liver of rat force-fed the wheat diet. Lipid is prominent in hepatic cells in periportal areas. Oil red O stain. × 66.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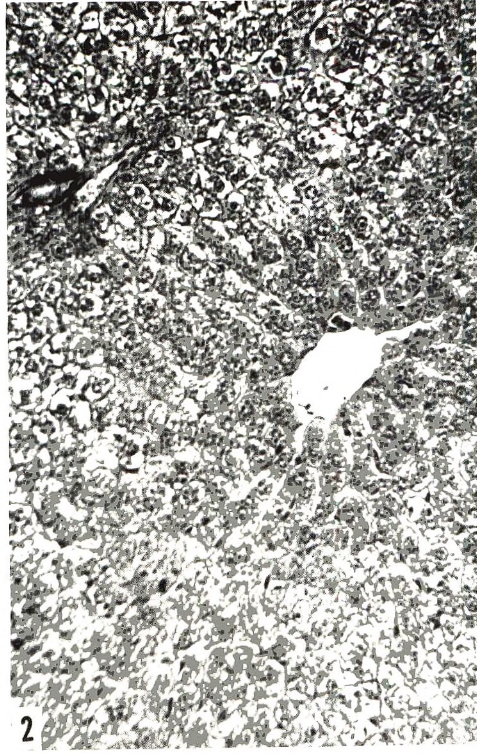
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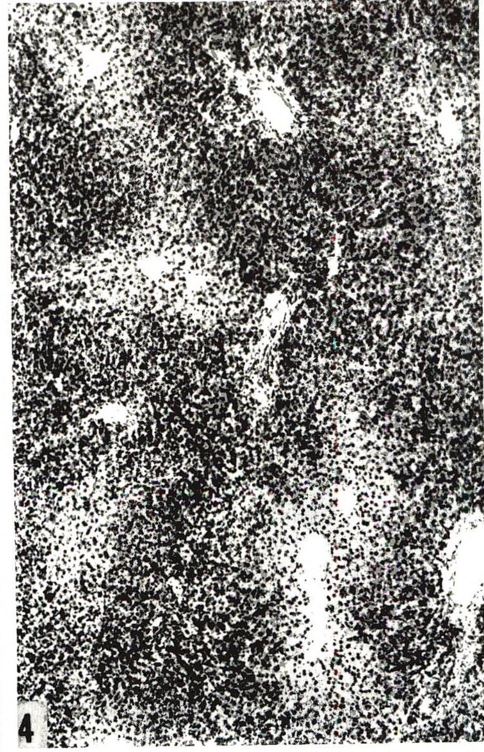
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PLATE 2

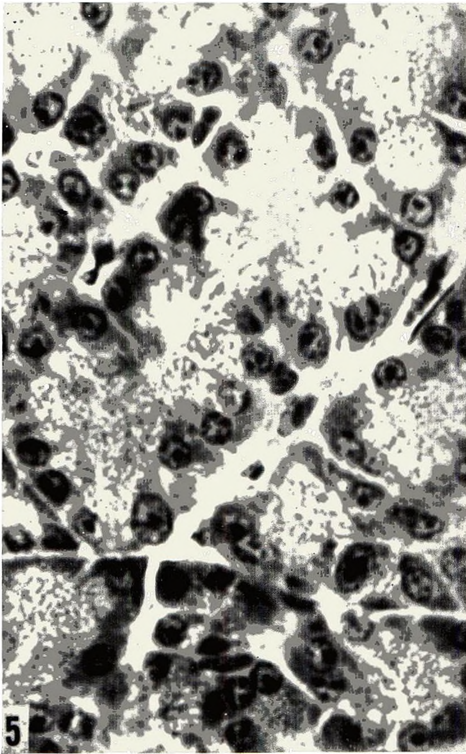
EXPLANATION OF FIGURES

5 Pancreas of control stock diet fed rat. Note abundant zymogen granules and size of cells. Hematoxylin and eosin. × 800.

6 Pancreas of rat force-fed the corn diet. Note reduction of zymogen granules and of cytoplasm of acinar cells. Hematoxylin and eosin. × 800.

7 Submaxillary gland of control stock fed rat. Hematoxylin and eosin. × 800.

8 Submaxillary gland of rat force-fed the corn diet. Note loss of cytoplasm of acinar cells. Compare with figure 7. Hematoxylin and eosin. × 800.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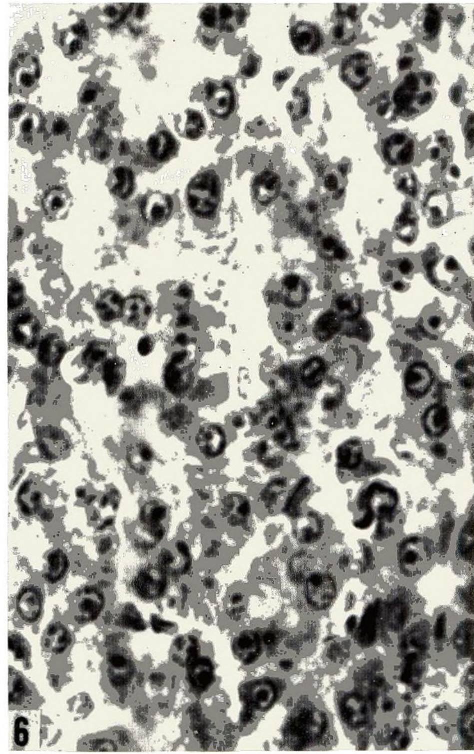
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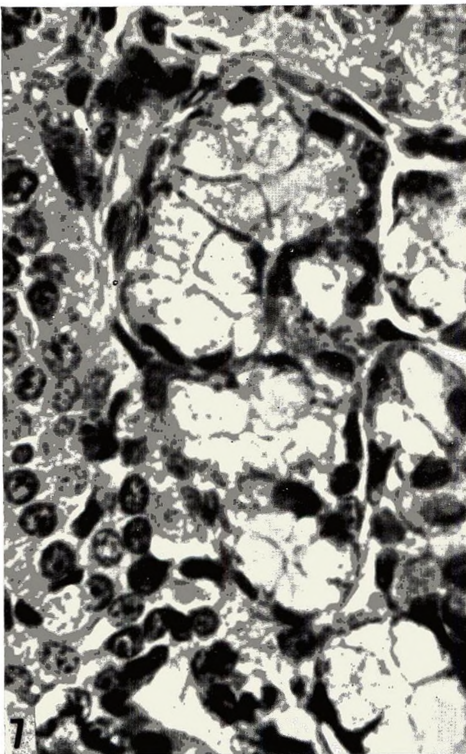
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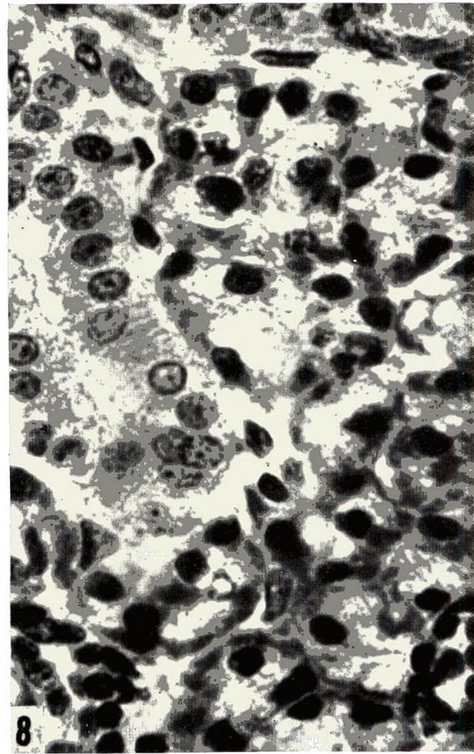
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Medium-chain and Long-chain Saturated Triglycerides and Linoleic Acid Requirements

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The nutritional properties of long-chain (C_{12-18}), saturated triglycerides have been shown to differ in many respects from those of medium-chain (C_{6-12}), saturated triglycerides (Kaunitz et al., '59). Ample evidence has been presented that long-chain triglycerides increase requirements for linoleic acid (Evans and Lepkovsky, '32; Deuel et al., '55; Barnes et al., '59); but there is some evidence that this is not true of medium-chain triglycerides. Work with the latter has been complicated by the presence of small amounts of linoleate in the samples of medium-chain triglycerides which were available.

Recently, triglycerides free of unsaturated acids have been prepared, and feeding of these materials has led to the clarification of some of the nutritional effects of long- and medium-chain triglycerides. The results of these studies as to body and organ weights of rats fed purified diets containing these materials and various levels of linoleic acid are reported below.

MATERIALS AND METHODS

The medium-chain (MCT) and long-chain (LCT), saturated triglycerides were prepared from coconut and other palm kernel oils by fractionation of the split fatty acids and reconstitution of the desired fractions into triglycerides after their compositions were checked by gas-liquid chromatography. The conversion was accomplished by reacting the fatty acid fractions with molar ratios of glycerol until the amounts of free fatty acid and glycerol were below 5%. The crude triglyceride fractions were alkali-refined, deodorized and filtered and then subjected to a vigorous stream of hydrogen under pressure in the presence of 0.3% nickel catalyst (0.1% nickel) at a temperature of 180 to 200°C for several hours. The resulting saturated material was cooled, filtered of the catalyst, decolorized by steam and refiltered to give an edible grade oil.

The MCT represented about 15% of the original coconut oil and was a clear, thin, odorless liquid with a melting point below 0°C and an iodine number of zero. It contained essentially no oleate or linoleate.

The LCT represented about 60% of the original coconut oil and was a solid fat having a melting point of about 40°C and an iodine number of zero. It contained less than 0.2% oleate and essentially no linoleate.

The studies were carried out with male albino rats from a well-established colony of the Sherman strain. Pregnant females from this colony were placed, approximately 4 days before birth of the young, on the "fat-free" (FF) diet given in table 1. Mothers and litters received this diet until the young were given the experimental diets at weaning. This change was made to partially deplete the young of linoleic acid (LA). Litters were pooled within three days of their birth and redistributed so that each mother received 6 small, 7 to 8 medium or 9 large male young to obtain more uniform rats. At 27 days, the young were weaned, earmarked and weighed. At 31 days, they were reweighed and distributed into 9 matching groups of 8 each, the average weights of which were equal at 24 days and again at 29 days. They then received the experimental diets described below. At 103 days, they were killed and their organs weighed.

The experimental diets included the FF diet given in table 1 with supplements

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of zero, 0.08 and 2% of a linoleic acid concentrate derived from safflower oil, and corresponding diets containing 20% of MCT or LCT added at the expense of the carbohydrate. The diets with 20% of fat contained 0.1% of linoleic acid concentrate instead of the 0.08% to compensate for the lower intake of these calorically richer diets. With 2%, no correction was made. Inasmuch as the alcohol-washed casein contained not more than 0.5% of fat and milk fat contains about 4% of LA, a daily intake of 10 to 15 gm of the diet without added LA would have afforded not more than 1 mg of LA—and probably less because of oxidation during preparation and storage of the casein.

For the evaluation of organ-weight data, log-log plots of organ weight against body weight were used. This was done because the ratio of the weight of an organ to the corresponding body weight is not linear but varies continuously, and, for most organs, a log-log plot gives a straight line distribution which may have one or more changes in slope with increasing body weight. The distribution has a uniform spread throughout its range. The normal organ-weight distributions used for comparison in these experiments were derived from organ-weight data collected, over a period of 4 years, from 427 male rats fed a diet similar to the experimental ones but containing 10 to 20% of fresh lard as fat. The animals weighed from 60 to 600 gm at the time of sacrifice but were grouped according to body weight so that the body weight range of each subgroup was small. The weights of an organ of each subgroup were averaged and the average was plotted against the body weight representing the midpoint of the group range. When data from all subgroups had been plotted, a straight line resulted which had the same slope as the distribution of all organ weights. This line became the source of the "normal" weight of an organ for any given body weight. The differences between the observed weights of an organ of an experimental group and their "normal" weights as derived from the appropriate line were expressed as percentages of the normal weights. These percentages of deviation from normal were averaged for each group and used for comparison between groups[1] and for t test analysis. In the latter, a P of 0.05 was considered to be just on the borderline of significance. Two experimental series were carried out; the results were in good agreement and have been combined.

TABLE 1

Composition of fat-free diet[1]

	%
Alcohol-washed casein[2]	30
Dextrose[3]	64
Salt mixture, U.S.P. 13	3.5
Ca carbonate	0.5
Cellulose[4]	2
Vitamins	*mg/kg*
Choline dihydrogen citrate	1000
Inositol	1000
p-Aminobenzoic acid	300
Nicotinamide	100
Vitamin K[5]	10
Thiamine·HCl	2
Pyridoxine·HCl	4
Riboflavin	4
Ca pantothenate	10
Folic acid	2.5
Biotin	0.025
Vitamin B_{12} (0.1% trituration)	5
Ascorbic acid	25
α-Tocopheryl acetate	100
Free α tocopherol	20
β-Carotene	10
Vitamin D_2	0.5

[1] We wish to thank Dr. Leo Pirk of Hoffman-LaRoche Inc., Nutley, New Jersey, for kindly supplying most of the vitamins used in the diets, and Dr. M. L. Tainter of the Sterling-Winthrop Research Institute, Rensselaer, New York for the vitamin D_2.

[2] General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Cerelose, Corn Products Refining Company, New York.

[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[5] Synkayvite, Hoffman-LaRoche Inc., Nutley, New Jersey.

EXPERIMENTAL

Figure 1 shows that the body weights of rats in all groups fed diets low in LA were depressed. There was little difference between corresponding groups fed MCT and FF diets, but with LCT, weights were depressed much more. When 2% of LA was added to the diets, the animals in the three groups grew equally well.

[1] The actual lines and a discussion of the method are given in Kaunitz et al., 1960 J. Nutrition, 70: 571.

LINOL. ACID SUPPLEM.	FF+ NO.	FF+ .08	FF+ 2%	MCT+ NO.	MCT+ 0.1	MCT+ 2%	LCT+ NO.	LCT+ 1.0	LCT+ 2%
BODY WGT grams	246 ±6.1	308 ±9.2	340 ±6.5	230 ±6.1	302 ±8.2	327 ±7.2	159 ±4.1	235 ±6.5	345 ±8.0
LIVER % weight deviation from normal (±)		±1.9	±1.7	±1.8	±1.4	±2.4	±2.4	±2.1	±1.4
LIVER ABS. WGT. grams	9.1 ±.25	9.4 ±.26	10.0 ±.24	9.3 ±.20	10.4 ±.21	9.8 ±.32	7.3 ±.32	9.3 ±.27	9.8 ±.30
KIDNEYS % weight deviation from normal (±)	±2.6	±2.2	±2.1	±2.6	±1.6	±1.7	±4.8	±2.0	±2.2
KIDNEYS ABS. WGT. grams	2.1 ±.05	2.0 ±.06	2.2 ±.06	2.0 ±.03	2.0 ±.05	2.2 ±0.4	1.6 ±.06	1.7 ±0.3	2.1 ±.04
TESTICULAR FAT BODIES % weight deviation from normal (±)		±4.9	±4.5	±5.8	±5.7	±5.0	±6.1	±6.3	±5.5
TESTICULAR FAT BODIES ABS. WGT. grams	2.9 ±.17	3.4 ±.17	4.0 ±.36	3.0 ±.17	4.1 ±.28	3.5 ±.19	1.8 ±.10	3.7 ±.20	4.9 ±.42

LINOL. ACID SUPPLEM.	FF+ NO.	FF+ .08	FF+ 2%	MCT+ NO.	MCT+ 0.1	MCT+ 2%	LCT+ NO.	LCT+ 1.0	LCT+ 2%
BODY WGT grams	246 ±6.1	300 ±9.2	340 ±6.5	230 ±6.1	302 ±8.2	327 ±7.2	159 ±4.1	235 ±6.5	345 ±8.0
HEART % weight deviation from normal (±)	±1.9	±1.8	±1.6	±1.8	±1.6	±2.1	±2.1	±1.8	±2.0
HEART ABS. WGT. grams	.72 ±.017	.72 ±.017	.81 ±.013	.72 ±.014	.80 ±.019	.81 ±.02	.54 ±.018	.69 ±.022	.86 ±.011
ADRENALS % weight deviation from normal (±)	±3.7	±4.5	±2.8	±3.8	±4.8	±6.1	±5.3	±5.2	±4.3
ADRENALS ABS. WGT. grams	.032 ±.0014	.033 ±.0015	.034 ±.0010	.031 ±.0009	.034 ±.0015	.040 ±.0018	.036 ±.0013	.042 ±.0015	.041 ±.0017
THYMUS % weight deviation from normal (±)	±5.5	±8.0	±5.0	±2.6	±5.5	±6.8	±1.6	±3.1	±5.7
THYMUS ABS. WGT. grams	.26 ±.023	.29 ±.027	.35 ±.016	.22 ±.011	.26 ±.017	.30 ±.026	.17 ±.009	.27 ±.019	.36 ±.014

Fig. 1 Body weights, organ weights and percentages of deviation from normal of the organs of male rats fed purified diets containing no fat, 20% MCT or 20% LCT and various levels of linoleic acid. Standard errors are indicated by ±.

The weights of all organs except the thymus were, in general, higher in relation to body weight in those groups given no LA, but were also affected by the triglycerides in the diet. Liver weights were somewhat higher in the LA-deficient groups fed MCT and LCT; but, with 2% of LA, there were no differences. Heart ventricle weights were relatively higher in the groups fed zero and 0.1% of LA when MCT and LCT were included in the diets. When 2% of LA was fed, LCT led to higher heart ventricle weights than MCT or the FF diet.

Kidneys of all groups fed no LA were enlarged; addition of 0.1% of LA reduced them to the point where 2% of LA had no further effect. The presence of triglycerides in the diet did not change this.

Adrenal weights were strongly affected by the kind of triglycerides fed. At levels of zero and 0.1% of LA, LCT led to adrenals which were relatively and absolutely heavier than those of the MCT and

FF groups. With MCT, adrenals were not different from those of the groups fed FF diets except at a LA level of 2%. That the absolute weights of the adrenals of rats fed LCT were higher is the more remarkable because their body weights were much lower than the weights of those in the corresponding MCT and FF groups. Addition of 2% of LA to the LCT diet led to adrenals of the same weight as those of the MCT group.

Testicular fat bodies were weighed because their weight has been shown to be proportional to the total neutral fat content of the rat (Hausberger, '37; Stoerk and Porter, '50). There were no significant differences between corresponding groups fed FF and MCT diets, but increasing supplements of LA led to smaller fat bodies. Feeding of LCT led to significantly enlarged fat bodies, which were not affected by 0.1% of LA.

Thymus weights were equally subnormal for all groups given no LA; addition of 2% of LA raised them significantly. Supplementation with 0.1% had a significant effect with the FF and MCT diets but not when fed with LCT.

DISCUSSION

One may ask whether the organ-weight changes of the LA-deficient groups were merely the result of inanition. A control group of 34 male rats was maintained on a purified diet containing 20% of lard. At 91 days of age, when the average weight of the rats was 332 gm, food was gradually withdrawn so that, after one week, they weighed 283 gm. At this weight they were maintained by restricted feeding until killed at 124 days. The percentages of deviation from normal of their organ weights (± standard error) are given in table 2. The data as to liver, kidneys, adrenals, thymus and heart are in good agreement with previous findings (Jackson, '25). The effect on the testicular fat bodies seemed to be dependent on the amount and duration of food restriction. In this experiment, the testicular fat bodies deviated very little from normal. Similar values were observed when other animals fed the same diet were restricted to constant weight without preliminary weight reduction. However, in a group of 16 rats, the weight of which had been more severely reduced, from 450 to 350 gm, and which had received restricted food intake for a longer period (46 days), the testicular fat bodies were 34% ± 4.1 smaller than in normal animals.

TABLE 2

Percentages of deviation from normal of organs of adult male rats after reduction of their body weight by restricted feeding and maintenance of this reduced weight for 33 days

Organ	Deviation from normal
	%
Liver	−37 ± 1.6[1]
Kidneys	−10 ± 1.3
Adrenals	+20 ± 3.0
Heart ventricles	−12 ± 1.6
Thymus	−54 ± 2.5
Testicular fat bodies	− 3 ± 4.1

[1] Standard error.

The only organ-weight changes associated with depressed body weight which were similar to those found in the LA-deficient groups were of the adrenals and thymus. All other changes were in the opposite direction. Thus, it appears that the organ-weight changes seen in LA deficiency are not the result of partial starvation.

These studies confirm earlier observations that feeding of long-chain, saturated acids increases LA requirements. However, this is not true of medium-chain triglycerides. Inclusion of these fats in a LA-deficient diet led to essentially the same changes as those observed when a fat-free diet was fed. Such signs of stress as low thymus weights and enlargement of other organs (especially the adrenals) were made worse by LCT but not by MCT.

Medium- and long-chain triglycerides have been shown to have different effects under other pathological conditions. In choline deficiency, fatty livers were not observed when the dietary fat consisted of triglycerides of medium chain length (Gey et al., '55). When included in a diet containing autoxidized cottonseed oil, MCT partially counteracted the toxicity of the rancid fat, whereas LCT aggravated the condition (Kaunitz et al., '60).

Whether all of these instances of the opposite nutritional effects of MCT and

LCT have one aspect in common (perhaps some relation to linoleic acid?) or whether the mode of action is different in each case remains for further study.

SUMMARY

1. Weanling male rats were maintained for 74 days on a purified, fat-free (FF) diet or similar ones containing 20% of saturated medium-chain (C_{6-12}) triglycerides (MCT) or long-chain (C_{12-18}) triglycerides (LCT). The diets were supplemented with zero, 0.1 or 2% of linoleic acid (LA).

2. Body weights of animals in the LA-deficient groups were similarly depressed with corresponding FF and MCT diets and more so with the LCT rations; with 2% of LA, rats in all groups had similar weights.

3. The deviations from normal of the organs in all LA-deficient groups included enlarged livers, adrenals, kidneys, heart ventricles and testicular-fat bodies. Thymus weights were reduced. Significant differences between corresponding LA-deficient groups fed the different triglycerides were noted; those fed MCT were usually similar to those receiving FF diets, whereas those fed LCT showed more pronounced deviations from normal.

4. It was concluded that MCT does not increase LA requirements in comparison with an FF diet and that LCT does.

5. Maintenance of control animals receiving reduced food intake led to organ-weight changes different from those found in LA deficiency: smaller livers, kidneys and heart ventricles than in normal animals and normal testicular-fat-body weights. This suggested that the organ-weight changes observed in the LA-deficient animals were not the result of partial inanition.

ADDENDUM

A more quantitative measure of the relative linoleic acid (LA) requirements of rats fed medium-chain (MCT) and long-chain (LCT) saturated triglycerides was established with two groups of 50 weanling males which had been born of and reared by mothers receiving the fat-free diet plus 0.1% of linoleic acid. At weaning, one group was given the MCT diet plus 0.1% of LA and the other was given the LCT diet plus 0.3% of LA. After two weeks, the average weight of the animals fed LCT was 118 gm and of those fed MCT, 124 gm. At this point, the LA level of the LCT diet was raised to 0.5%. After 20 days at the new level, the average weight of those fed LCT was 197 gm and of those fed MCT plus 0.1% of LA, 195 gm. Nine days later, the LA supplement of the LCT diet was reduced to 0.3%. After 5 days, the animals fed LCT weighed 235 gm and those fed MCT weighed 233 gm. This suggests that, with a marginal LA intake, the requirements of rats fed LCT were about 4 times those of the rats fed MCT.

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Lactose Diets and Cholesterol Metabolism

I. CHOLESTEROL ABSORPTION, COPROSTANOL FORMATION AND BILE ACID EXCRETION IN THE RAT[1]

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Lactose-supplemented diets increase cholesterol absorption in the rat when measured by the difference between cholesterol intake and excretion and by elevated liver-cholesterol concentration (Wells and Cooper, '58; Nath et al., '59). These findings are based on the assumption that destruction of cholesterol by intestinal micro-organisms is insignificant (Wainfan et al., '52; Stadtman et al., '54), and that liver-cholesterol synthesis is not affected by dietary lactose. Since quantitative absorption of cholesterol into the thoracic lymph has been reported by several laboratories (Bollman and Flock, '51; Chaikoff et al., '52), the direct measurement of administered cholesterol-4-C^{14} by means of lymph cannulation in rats fed lactose- or sucrose-containing diets was carried out, and the results confirm our previous observations. Several possible explanations have been presented for the cholesterol absorption stimulating effect of lactose (Wells and Cooper, '58; Wells and Anderson, '59). One of these, the inhibition of the conversion of cholesterol to coprostanol which is poorly absorbed (Behring and Schoenheimer, '30) has been considered. Recently, the most promising finding for an effect of lactose is the observation that the excretion of bile acids from bile duct-cannulated rats previously fed a lactose-containing, cholesterol-free diet is significantly higher than in the sucrose-fed controls.

METHODS AND EXPERIMENTAL

Lymph cannulation

Male rats of the Long-Evans strain weighing 250 to 300 gm were divided into two groups of 5 and fed diets which varied only in carbohydrate content. The sucrose control diet consisted of the following (per cent): sucrose, 62.8; casein, 18; Wesson salts, 4; cottonseed oil, 15; choline chloride, 0.1; vitamin mixture, 0.1; and ample supplements of α-tocopheryl acetate in cod liver oil. The second diet contained 40% of lactose at the expense of sucrose. The rats were fed the respective diets for 5 days at which time a polyethylene cannula[2] was inserted into the thoracic lymph duct according to the procedure of Bollman et al. ('48). While the animals were still under light ether anesthesia, a meal of 27.2 mg of cholesterol-4-C^{14} [3] (153,000 cpm) dissolved in 1 ml of cottonseed oil was administered by stomach tube. The animals were placed in restraining cages and given saline during the 24-hour collection period. The lymph was collected in a flask containing 5 ml of 10% potassium oxalate. At the end of the collection period, the total lymph volume was recorded and a 4-ml aliquot was placed in a 50-ml volumetric flask containing ethanol: acetone (1:1). The extraction was carried out essentially as described by Daskalakis and Chaikoff ('55). The contents were made to volume with the same solvents, filtered, and two 20-ml aliquots of the filtrate were saponified followed by acidification with 10% acetic acid. The cholesterol was precipitated with 10 ml of a 0.5% digitonin solution. One of the washed aliquots was filtered onto a weighed filter paper and counted as the

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[2] Polyethylene medical tubing, PE-90 Clay-Adams, Inc., New York.
[3] Nuclear-Chicago Corporation.

digitonide with a gas flow thin end window counter. Corrections were made for self-absorption when necessary. The second aliquot was used to determine the quantity of cholesterol present according to the Sperry-Webb ('50) procedure.

Coprostanol formation

Three series of 4 to 6 male albino rats of the Holtzman strain weighing 250 to 300 gm were fed a basal sucrose diet identical with the control diet previously described, with the exception that 0.5% of cholesterol was added at the expense of sucrose. At the end of one week, the animals were killed, and the small intestines removed and cut into segments equivalent to the first half, third quarter and 4th quarter. The contents of each corresponding segment were carefully washed from the tissue with saline, and the combined material was saponified for 2 hours with 100 ml of 10% alcoholic KOH. The nonsaponifiable matter was chromatographed on silicic acid: Celite (2:1) columns and cholesterol and coprostanol determined by differential colorimetric analysis (Coleman et al., '56). In a collateral experiment, the colon and cecum were surgically removed from two adult male rats of the Holtzman strain (350 gm). The distal end of the ileum was anastomosed to the anus. The animals then received a diet consisting in per cent of sucrose, 70.65; casein, 18; Wesson salts, 4; glycerol, 5; brain spinal cord lipids,[4] 2, cholesterol, 0.25; vitamin mixture, 0.1; choline chloride, 0.1; and supplements of cod liver oil containing α-tocopheryl acetate for 12 days. Droppings were collected on brown paper, dried in vacuo and extracted with Skellysolve B (B.P. 60 to 65°C). The lipid extract was saponified and the nonsaponifiable fraction was extracted with ether. After removal of the solvent, the nonsaponifiable matter was analyzed for cholesterol and coprostanol as described above.

Bile acid excretion

Two groups of adult male rats of the Holtzman strain were fed a sucrose- or lactose-containing diet which was free of cholesterol. The control sucrose diet had the same composition as described for the lymph cannulation study. The experimental diet contained 40% of lactose at the expense of sucrose. Six rats received the sucrose control diet and 8 rats the lactose-containing diet for 5 days. The animals were placed under ether anesthesia, and the common bile duct was cannulated. Bile was collected in a closed flask during the following 24-hour period. The volume of bile was measured and 5 to 6 volumes of absolute ethanol were added to the flask and the contents heated on a steam bath for 15 minutes. The precipitated mucin was filtered from the solution and the filtrate quantitatively washed into a 100-ml volumetric flask. For the quantitative determination of cholic acid and chenodeoxycholic acid, 15 ml of the alcohol filtrate were placed in a 250-ml round-bottom flask and the ethanol removed in vacuo. Five milliliters of 1.25N NaOH were added and the mixture was autoclaved at 120° for three hours. The hydrolyzed bile salts were then acidified with concentrated HCl to a pH of 3.0, and extracted with diethyl ether as recommended by Mosbach et al. ('54). The ether extract was washed with water until the washings were negative to a silver nitrate solution. The ether was removed from the sample and the bile acids were dissolved in a few milliliters of outer-phase solvent (55% aqueous methanol) and chromatographed on a reversed phase partition chromatogram of silane[5]-impregnated Celite with chloroform: petroleum ether (9:1) as inner phase according to the description of Bergström and Sjövall ('51) and Sjövall ('53) and modified by Mirvish ('58). Samples (3 ml each) were collected in small cuvettes with the aid of a fraction collector and the acidity of each fraction was titrated electrometrically with 0.01N NaOH. Additional aliquots of the original filtrate were analyzed for cholesterol content by a modification of the procedure of Portman et al., ('55) using direct Liebermann-Buchard colorimetry on the separated sterol fraction.

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[4] A cerebroside-rich fraction generously supplied by Armour and Company, Chicago.

[5] Dimethyldichlorosilane, a generous gift of the General Electric Company, Cleveland.

RESULTS

Lymph cannulation

The average volume of the lymph secreted by the lactose-fed group was nearly twice that of the sucrose-fed con-controls (table 1, 128.0 ± 30.9 ml vs. 71.2 ± 31.6 ml). Similarly, the average recovery of cholesterol-4-C^{14} in the first 24-hour lymph collection was 19.6 ± 7.4 and 7.5 ± 1.4% for the lactose- and sucrose-fed rats, respectively (table 1). Analysis of the total cholesterol of the lymph revealed that the lactose-fed group secreted more cholesterol than the sucrose-fed controls (table 1, 15.7 ± 2.1 mg/24 hours vs. 9.8 ± 1.7 mg/24 hours). The dilution of the absorbed cholesterol-4-C^{14} by nonisotopic endogenous cholesterol was more pronounced in the sucrose-fed group (average specific activities: sucrose group, 1,216 cpm/mg; lactose group, 1,947 cpm/mg).

Intestinal coprostanol formation

The results of direct isolation and analysis of coprostanol from different segments of the small intestine and cecum (table 2) regularly demonstrated a significant quantity of this stanol in the cecum, lower fourth, and third quarters of the small intestine and small but measurable amounts in the first half of the small intestine in most instances (table 2, experi-

TABLE 1

The effect of lactose feeding on the recovery of cholesterol-4-C^{14} in thoracic duct lymph of the rat[1]

Diet[2]	Lymph flow	Total sterol recovered in 24-hour lymph	Cholesterol-4-C^{14} recovered in 24-hour lymph	Av. specific activity of recovered cholesterol-4-C^{14}
	ml	*mg*	*% of administered*[3]	*cpm/mg*
Sucrose	71.2 ± 31.6[4]	9.8 ± 1.7[4]	7.5 ± 1.4[4]	1,216
Lactose	128.0 ± 30.9	15.7 ± 2.1	19.6 ± 7.4	1,947

[1] Each group consisted of 5 adult male rats of the Long-Evans strain weighing 250 to 300 gm.

[2] See text for composition.

[3] Given orally: 27.7 mg of cholesterol-4-C^{14} (153,000 cpm) dissolved in 1 ml of cottonseed oil.

[4] Standard deviation, $\sigma = \sqrt{\Sigma X^2 - \frac{(\Sigma X)^2}{N} / N - 1}$

TABLE 2

Distribution of cholesterol and coprostanol in the intestinal lumen of cholesterol-fed rats[1]

Experiment[2]	Intestinal segment	Combined weight of sterol: Cholesterol	Combined weight of sterol: Coprostanol	Coprostanol × 100 / Total sterol [3]
No.		*mg*	*mg*	*%*
1	First half	1.29	0.15	10.0
	Third quarter	1.13	1.71	58.2
	Fourth quarter	9.70	10.00	46.8
	Cecum	14.50	36.60	66.2
2	First half	2.50	1.60	28.2
	Third quarter	0.40	1.08	39.5
	Fourth quarter	7.10	2.40	23.1
	Cecum	11.10	38.00	71.5
3	First half	0.11	1.62	78.0
	Second half	1.98	3.10	56.0
	Cecum	21.10	65.30	66.3

[1] Experiments 1 and 2 each consisted of 4 rats (250 to 300 gm), experiment 3 consisted of 6 rats (250 to 300 gm).

[2] See text for diet composition.

[3] Corrected to include Δ^7-cholestenol and methostenol.

ments 2 and 3). Removal of the cecum and colon (two male adult rats) did not eliminate the ability to convert appreciable amounts of cholesterol to coprostanol (22.2 and 38.9% of total fecal sterols in the two collection periods, respectively). Normal rats consuming the same diets as the operated rats excreted sterol mixtures containing 22.3 and 74.2% of coprostanol during the same collection periods.

Biliary bile acids and cholesterol

The average volume of bile collected from each group of rats was not significantly different (table 3, sucrose, 13.6 ml; lactose, 14.8 ml). However, the average total cholic acid excretion in the first 24 hours after cannulation was approximately 70% higher in the lactose-fed group than in the sucrose-fed controls (table 3, 75.3 ± 9.8 mg/24 hours vs. 44.2 ± 5.6 mg/24 hours, respectively). In contrast, the excretion of chenodeoxycholic acid was similar in both groups (table 3, sucrose, 5.5 ± 1.2 and lactose, 4.8 ± 2.1 mg/24 hours). Three rats previously fed lactose but not given food during the cannulation period excreted somewhat less bile acids than those given food during this period (table 3, 70.2 mg vs. 80.1 mg). The average cholesterol excretion by these animals was notably high (3.89 mg/24 hours) compared with the cholesterol excretion of the sucrose- and lactose-fed animals given access to ration during the collection period (1.84 and 1.65 mg/24 hours, respectively).

DISCUSSION

The direct demonstration of an increase in cholesterol absorption has been possible in the rat by means of lymph collection, but similar studies in the rabbit and other species remain to be conducted. Temporarily, one may assume that in the rabbit a similar event leads to the increased concentration of serum and liver cholesterol previously observed (Wells and Anderson, '59).

Several possible mechanisms for the lactose-stimulated cholesterol absorption exist (Wells and Anderson, '59). Our present findings tend to favor one or possibly two mechanisms operating either independently or concurrently. The question of the significance of the degree of coprostanol formation on cholesterol absorption requires information as to (1) the site of absorption of cholesterol and (2) the site of the formation of coprostanol. The extent of coprostanol formation as a mechanism can be given serious consideration only if the two sites overlap such that at least some of the coprostanol formed will pass along the active site of sterol absorption. Coprostanol which is not readily adsorbed from the intestines would tend to increase the excretion of the body sterol supply. The formation of coprostanol is well known to be inhibited by a lactose-containing diet (Müller, '00; Dorée and Gardner, '08; Dam, '34). By following the intestinal absorption pathway of dietary cholesterol-4-C^{14}, Swell et al. ('58) have shown that the site of cholesterol absorption is in the first half of the small intestine. The sterol analysis reported here reveals the presence of small but significant amounts of coprostanol in the first half of the small intestine. A more attractive mechanism for the lactose-diet effect

TABLE 3

The biliary excretion of bile acids and cholesterol by rats fed sucrose- or lactose-containing diets

Group[1,2]	Diet[3]	Average bile volume	Bile acids			Cholesterol
			Cholic	Chenodeoxy	Total	
		ml	*mg/24 hours*			*mg/24 hours*
1	Sucrose	13.5	44.2 ± 5.6[4]	5.5 ± 1.2[4]	49.7	1.84 ± 0.24[4]
2	Lactose	14.4	75.3 ± 9.8	4.8 ± 2.1	80.1	1.65 ± 0.41
3	Lactose	16.3	63.6(56.8–67.1)	6.6(5.8–8.1)	70.2	3.89(2.2–6.2)

[1] Groups 1 and 2 were fed during the 24-hour collection period whereas group 3 was not.
[2] Group 1, 2 and 3 consisted of 6, 5 and 3 male rats (230 to 250 gm) respectively.
[3] See text for diet composition.
[4] Standard deviation, $\sigma = \sqrt{\Sigma X^2 - \frac{(\Sigma X)^2}{N} / N - 1}$

is its marked influence on bile acid metabolism. There is an appreciable increase in biliary excretion of cholic acid in the 24-hour period following bile duct cannulation by rats previously fed a lactose-containing diet as compared with the sucrose-fed controls.

The data are consistent with the amount of bile salts which would be necessary to account for the increased cholesterol absorption seen in our previous studies using diets of the same composition (Wells and Cooper, '58). Since bile cannulation produces an unphysiological drain on the liver supply of bile acids (Ericksson, '57), the values we have obtained within the first 24 hours must be considered from the standpoint of either the release of stored high levels of bile acids (i.e. bile acid pool) or the potential capacity of the livers of each group to synthesize new bile acids from cholesterol. Studies are now in progress to determine the liver bile acid pool size in sucrose- and lactose-fed rats. Additional evidence of an effect on cholesterol metabolism by lactose diets can be found by a study of the rate of incorporation of radioactive acetate and mevalonate into the liver cholesterol of rats previously fed sucrose- or lactose-containing diets. Preliminary results using injected sodium acetate-2-C^{14} as the sterol precursor revealed that the specific activity of liver cholesterol from sucrose-fed rats was significantly higher than that from lactose-fed rats.[6] Studies are also in progress to determine the rate of cholesterol-4-C^{14} conversion to bile acids in the sucrose- and lactose-fed rats.

The studies of Lindstedt and Norman ('56) have indicated that in the normal rat, the half-life of the different common bile acids was about two days, whereas if antibiotics were fed, the half-life increased to approximately 10 days. If a lactose-containing diet is found to decrease the turnover rate of cholic acid in the rat,[7] it would follow that the environment of the intestines under the conditions of lactose stimulation, i.e. Lactobacillus-promoting, would favor reabsorption of the bile acids. The inhibition of the normal degradation of bile acids to nonabsorbable derivatives could be invoked as a possible explanation. The degradation of bile acids by certain unknown micro-organisms of the intestinal tract (Gustafsson et al., '57) could be an important controlling factor with regard to the net excretion of bile acids as suggested by Bergström ('59). Lactose-stimulated hypercholesterolemia in the rabbit (Wells and Anderson, '59) and similarly high liver concentrations of cholesterol in the rat (Wells and Cooper, '58) may result from the following sequence of events. The increased reabsorption of bile acids from the intestinal tract would result in an increased pool of circulating bile acids. Higher levels of circulating bile acids would influence the conversion of cholesterol to bile acids in the liver and increase the absorption of any exogenous source of cholesterol from the intestine.

SUMMARY

1. Rats were fed a meal of cholesterol-4-C^{14} and the cholesterol recovered in thoracic duct lymph during the first 24 hours was studied. The sucrose-fed animals absorbed an average of 7.5% of the isotope whereas rats fed a 40% lactose-containing diet absorbed 19.6% of the cholesterol under identical conditions.

2. Analysis of the sterol composition of various segments of the small intestine and of the cecum of cholesterol-fed rats revealed the presence of appreciable amounts of coprostanol in the cecum, fourth and third quarters of the small intestine, and of small but measurable quantities in the first half of the small intestine.

3. The quantity of cholic acid excreted through bile duct fistulas by a group of rats fed sucrose and lactose was found to be an average of 44.2 mg/24 hours and 75.3 mg/24 hours, respectively. The importance of the intestinal flora and the resulting effect on bile acid and cholesterol metabolism is discussed.

[6] Wells, W. W., S. C. Anderson and R. Quan Ma 1960 The effect of lactose-containing diets on sterol metabolism in the rat. Federation Proc., *19*: 237 (abstract).

[7] Without presenting their data, Portman and Stare ('59), have reported that rats fed lactose-containing diets have slower turnover rates of radiocholate than rats fed sucrose.

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The Interrelationship Between Vitamin B_6 and B_{12} Deficiencies in Rats[1]

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Although the biochemical changes accompanying severe deficiencies of vitamins B_6 and B_{12} have been subjects of many studies, few reports are available on the effect of deficiencies of one of these two vitamins leading to the deficiency of the other vitamin. Recently, it was demonstrated that vitamin B_6 deficiency brings about a partial impairment of the absorption of vitamin B_{12} by adult rats (Hsu and Chow, '57). If this phenomenon were true, it might be expected that prolonged vitamin B_6 deficiency in rats may result in the development of vitamin B_{12} deficiency. The purpose of this communication is to present our data on the effect of prolonged feeding of a vitamin B_6-deficient diet on the vitamin B_{12} reserves as measured by the plasma and hepatic B_{12} levels of the vitamin. Conversely, data will be presented on the effect of vitamin B_{12} deficiency on transaminase concentrations, an enzyme believed to be related to vitamin B_6 deficiency.

METHODS

Preparation of animals

With vitamin B_{12} deficiency. Male and female progenies of rats fed a soybean diet (Ling and Chow, '52) low in vitamin B_{12} content during pregnancy and lactation, were placed in individual cages with large screen bottoms, and offered the same soybean diet. For comparison, litter mates were fed the same basal diet supplemented with vitamin B_{12} (100 µg/kg of diet). These diets were offered to the two groups of animals for 6 to 8 months, to be certain that severe deficiencies of vitamin B_{12} developed in the group deprived of this vitamin.

With vitamin B_6 deficiency. Male weanling rats of the McCollum strain, as well as of the Wistar strain, were offered, ad libitum, the vitamin B_6-deficient diet consisting of casein, sucrose, minerals and all known vitamins except vitamin B_6, (Tulpule et al., '55). These animals were housed in screen bottom cages. For comparison, the litter mates were supplied daily (1) by the injection of 50 µg of pyridoxine hydrochloride, and (2) by the supplementation of the basal diet with pyridoxine (100 µg/kg). The animals were placed under these dietary regimens for 6 months, and the usual symptoms of vitamin B_6 deficiency such as acrodynia and loss of weight and appetite developed in the group deprived of this vitamin.

Methods of analysis

Serum glutamic oxalacetic acid transaminase activity. The serum glutamic oxalacetic acid transaminase activity (SGOT) was measured by the procedure of Steinberg et al. ('56) and was taken as a measure of vitamin B_6 deficiency.

Xanthurenic acid test. The normal metabolism of tryptophan is impaired as the result of vitamin B_6 deficiency, with a large increase in the excretion of xanthurenic acid. To perform a tryptophan load test, 50 mg of D,L-tryptophan per 100 gm of body weight of animals deficient in vitamin B_6 or those treated with it were given by mouth. Urine collection was made for 24 hours for the measurement of xanthurenic acid, according to the procedure of Rosen and Sprince ('51).

Vitamin B_{12} determinations. Vitamin B_{12} concentrations in sera or plasma were determined by the modified procedures of

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Okuda et al.,[3] the details of which have been published elsewhere (Gaffney et al., '57). Livers from experimental animals were homogenized in Potter-Elvehjem glass homogenizers with 10 times their weight of distilled water, treated with potassium cyanide and assayed microbiologically for vitamin B_{12} activity.

RESULTS

The effect of vitamin B_6 deficiency on vitamin B_{12} absorption. Fifty millimicrograms of radioactive vitamin B_{12} labeled with Co^{60} (B^*_{12}) were given by mouth to vitamin B_6-deficient and B_6-injected animals. The radioactivities in the 4 days' feces and various target organs are shown in table 1. These results demonstrate that deficient and treated animals absorbed 32 and 64% of the administered dose, respectively, and, therefore, confirm our previous findings that vitamin B_{12} absorption is impaired by vitamin B_6 deficiency.

The effect of vitamin B_6 deficiency on vitamin B_{12} reserves. Four groups of weanling rats were offered a basal diet deficient in vitamin B_6. Group A received the basal diet alone. To group B, only vitamin B_{12} was given as the supplement to the basal diet (50 μg/kg). Group C received the basal diet supplemented with both vitamin B_6 (5 mg/kg) and vitamin B_{12} (50 μg/kg). Group D received both vitamins B_{12} (50 μg/kg) and B_6 (100 mg/kg). Thus, group D diet contained 20 times more vitamin B_6 than the group C diet. After three months' feeding, the vitamin B_6-deficient animals (both groups A and B) had lower mean body weights than those animals (groups C and D) receiving the vitamin B_6 supplement (table 2). However, there was no statistically significant difference in favor of the higher dose of vitamin B_6. The vitamin B_{12} serum level of rats in group A was lower than that of those in group B which received supplementary vitamin B_{12}, showing that the ability of these animals to absorb was not completely destroyed. Despite supplementation with such a large dose of vitamin B_{12}, the serum levels of vitamin B_{12} in the latter group (B) was still considerably below those of groups C and D. Similarly, animals in group A deprived of both vitamins B_6 and B_{12} had the lowest concentration of B_{12} in the liver. The hepatic storage was increased when vitamin B_{12} was added to the diet in group A and was further increased when a moderate amount of vitamin B_6 was also provided in the

[3] Okuda, K., R. D. Wood, C. A. Lang and B. F. Chow 1954 Serum levels of vitamin B_{12} in man. Federation Proc., *13:* 471 (abstract).

TABLE 1

Effect of vitamin B_6 deficiency on vitamin B_{12} absorption

Treatment	Feces	B_{12} absorbed	Liver vitamin B^*_{12}[1]	Kidney vitamin B^*_{12}[1]
	mμg	*mμg*	*mμg*	*mμg*
Vitamin B_6-injected (6)[2]	18	32 ± 2.6	4.4 ± 0.2	3.3 ± 0.1
Vitamin B_6-deficient (6)	34	16 ± 2.4	2.9 ± 0.3	2.7 ± 0.2

[1] Fifty millimicrograms of vitamin B^*_{12} (radioactive B_{12} labeled with Co^{60}) was given by mouth.

[2] Numbers in parentheses denote number of rats used.

TABLE 2

Effect of vitamin B_6 deficiency on vitamin B_{12} levels in serum and in liver

Group	Treatment		Body weight	Vitamin B_{12} in serum	Vitamin B_{12} in liver
	Vitamin B_6	Vitamin B_{12}			
			gm	*μμg/ml*	*mμg/gm*
B (10)[1]	—	+	164 ± 8	656 ± 51	335 ± 11
C (10)	+	+	268 ± 15	1020 ± 68	464 ± 30
A (10)	—	—	165 ± 8	132 ± 20	196 ± 27
D (10)	++	+	281 ± 16	1105 ± 129	262 ± 25

[1] Numbers in parentheses denote number of rats used.

same diet (compare groups B and C). However, in contrast the hepatic level of vitamin B_{12} was considerably reduced when a large amount of vitamin B_6 was incorporated in the diet in group D. The interplay between concentrations of vitamins B_6 and B_{12} can also be seen from the results to be described later. Since vitamin B_6 deficiency could affect the absorption of vitamin B_{12}, one might inquire conversely whether vitamin B_{12} deficiency would likewise result in some abnormalities of enzyme systems associated with vitamin B_6 deficiency. Therefore, the plasma SGOT level of vitamin B_{12}-deficient animals was determined.

The effect of vitamin B_{12} deficiency on SGOT. Male and female rats were made deficient in vitamin B_{12} by offering them the basal soybean diet deficient in vitamin B_{12} for 6 months. The results shown in table 3 demonstrate the degree of deficiency by the low serum and hepatic levels. It is interesting to note that the animals deficient in vitamin B_{12} showed considerably higher SGOT concentration, which is probably due to some damage to transaminase-rich organs such as liver. The elevation of this enzyme is particularly marked in the male animals which are more susceptible to vitamin B_{12} deprivation than the females, as judged by retardation of growth rates.

The effect of vitamin B_{12} injection on SGOT of vitamin B_6-deficient rats. Since the above data demonstrate interrelationship between vitamin B_{12} and B_6 deficiencies, it was also of interest to determine whether the administration of vitamin B_{12} to animals deficient in vitamin B_6 would alter the SGOT level. To this end, vitamin B_{12} (5 μg/day/rat) was injected into vitamin B_6-deficient and B_6-treated animals. Their SGOT levels were compared with those receiving neither vitamins B_6 nor B_{12}. It can be seen from table 4 that the injection of vitamin B_{12} had no effect on the SGOT level; whereas, supplementation with vitamin B_6, as one would expect, increased the serum level of this enzyme.

Interplay between vitamin B_{12} and B_6. Data presented in table 2 indicate that supplying vitamin B_6 in greater abundance brought about a marked decrease in the

TABLE 3

The SGOT[1] level of vitamin B_{12}-deficient animals

Group	Body weight	Liver	Serum B_{12}	Liver B_{12}	Total liver B_{12}	SGOT
	gm	*gm % body weight*	*μμg/ml*	*mμg/gm liver*	*mμg/% body weight*	*units/ml*
Vitamin B_{12}-deficient (13 males)[2]	211.3 ± 18[3]	5.20 ± 0.33	114.6 ± 10.1	24.5 ± 6.4	121.9 ± 12.7	116.6 ± 8.6
Vitamin B_{12}-supplemented (13 males)	328.0 ± 20	3.43 ± 0.01	1372.0 ± 50.3	150.8 ± 14.2	542.1 ± 59.3	77.3 ± 2.9
Vitamin B_{12}-deficient (10 females)	233.9 ± 4	3.13 ± 0.10	65.3 ± 7.0	56.9 ± 4.8	176.9 ± 14.1	84.0 ± 3.7
Vitamin B_{12}-supplemented (10 females)	231.5 ± 6	3.06 ± 0.14	954.1 ± 37.5	328.7 ± 19.3	1009.7 ± 79.2	62.1 ± 2.5

[1] Serum glutamic oxalacetic acid transaminase activity.
[2] Numbers in parentheses denote number of rats used.
[3] Standard error of the mean.

TABLE 4
Injection of vitamin B_{12} and SGOT[1] levels in serum

Group	Treatment		SGOT
	B_6	B_{12}	
			units/ml
A (12)[2]	—	—	23.9 ± 0.8
B (12)	—	+	26.8 ± 2.2
C (10)	+	+	68.5 ± 5.3

[1] Serum glutamic oxalacetic acid transaminase activity.
[2] Numbers in parentheses denote number of rats used.

hepatic vitamin B_{12} level, as expressed both in the amount of B_{12}/gm of liver, or total B_{12} in the entire organ, per 100 gm of body weight. Data given in table 5 demonstrate further that a larger supply of vitamin B_6 did not increase the SGOT level, and as a matter of fact brought about a significant decrease of this enzyme and a slight increase in xanthurenic acid upon a tryptophan load test (compare C and D groups).

DISCUSSION

The procedure often employed to study the effect of acute vitamin deficiency is to feed the experimental animals a diet containing all the known vitamins in sufficiently large quantities, except the one under investigation. It is assumed that the vitamins provided in the diet will meet the so-called normal requirements on the premises that deficient animals will have a normal dietary intake, with no impairment of absorption. Unfortunately, neither assumption is true since the deficient animals usually consume lesser amounts of the experimental diet. In this communication, we have brought forth the fact that in addition to the lower intake, deficiency of one vitamin, e.g., B_6, may bring about poorer absorption of a second one (vitamin B_{12}). If the poorer absorption is allowed to take place for a prolonged period, one may expect animals to become deficient in vitamin B_{12}. This was found to occur in the vitamin B_6-deficient animals, which were offered for a prolonged period a diet low in vitamin B_6, even though the diet contained unusually large amounts of vitamin B_{12}. Also noteworthy is that in the animals receiving excessive amounts of vitamin B_6, that is, a quantity considered far above the daily requirement for rats, a marked decrease in vitamin B_{12} serum level in liver and plasma was observed. Furthermore, the supplementation with a large amount of vitamin B_6 will decrease the transaminase level in plasma. These observations are suggestive of the importance of balanced intake of the vitamins.

The present study was not intended to throw additional light on the mechanism of vitamin B_6 deficiency which brings about the impairment of absorption. It might be worthwhile to mention the work of Yeh et al. ('59), who demonstrated that vitamin B_6 deficiency brought about a decrease in the secretion of gastric juice and intrinsic factor as well as an increase in the gastric acidity. However, these investigators pointed out further that vitamin B_6 deficiency also causes dysfunction of adrenals. These findings merely emphasize our thesis that deficiency of one vitamin can create disturbances in endocrines, and abnormal utilizaton of other vitamins besides the one being studied.

TABLE 5
The intake of vitamin B_{12} and B_6 on SGOT[1] levels

Group	Treatment		SGOT	Xanthurenic acid
	Vitamin B_6	Vitamin B_{12}		
			units/ml	*mg % / body weight*
B (10)[2]	—	+	32.1 ± 4.1	12.5 ± 1.4
C (10)	+	+	86.5 ± 5.0	0.5 ± 0.2
A (10)	—	—	23.9 ± 0.8	10.9 ± 1.1
D (10)	++	+	66.0 ± 4.2	1.17 ± 0.2

[1] Serum glutamic oxalacetic acid transaminase activity.
[2] Numbers in parentheses denote number of rats used.

SUMMARY

Data are presented to show that feeding rats a diet deficient in vitamin B_6 will result in the lowering of vitamin B_{12} reserve as manifested by the low B_{12} serum level and hepatic storage. Oversupply of vitamin B_6 likewise lowers the vitamin B_{12} reserve in liver, perhaps due to increased metabolic requirement. Vitamin B_{12} deficiency in rats brings about an elevation of transaminase activity in serum, a measure commonly used to estimate the state of sufficiency of vitamin B_6. The significance of the interplay between vitamins is discussed.

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The Relative Effects of Pyridoxine Deficiency on Two Plasma Transaminases in the Growing and in the Adult Rat[1]

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The relationship between pyridoxal phosphate (B_6P) and transaminase enzymes is now well established, and numerous papers have appeared which demonstrated that tissue transaminase activity was depressed in pyridoxine-deficient experimental animals[2] (Brin et al., '54; Caldwell and McHenry, '53; Schlenk and Fisher, '47). Most of the early work was confined to the aspartic enzyme. Where both enzymes were assayed in pyridoxine-deficient animals under controlled conditions, it appeared that the alanine enzyme was more markedly depressed than the aspartic system[3] (Brin et al., '54; Caldwell and McHenry, '53). It is of interest that the alanine enzyme was also markedly affected in tissues after total body X-irradiation, fasting, and cortisone administration possibly demonstrating a greater sensitivity of this transaminase to physiological change (Awapara, '53; Brin and McKee, '56; Brin et al., '54).

The question remained as to whether serum transaminases were affected in the same manner as the tissue transaminases in this deficiency. Early studies with monkeys (Marsh et al., '55) demonstrated variable transaminase values although the relationship was established. A number of studies with human subjects have also demonstrated a positive, though variable, relationship between pyridoxine deficiency and the serum aspartic enzyme (Sass and Murphy, '58; Vilter et al., '53). We have now shown that in rat plasma the relationship was definitive. Similar to the situation in duck heart, the alanine enzyme was more sensitive than the aspartic enzyme. The purpose of this paper is to elaborate upon the earlier report.[4] This concept has recently been confirmed by Babcock ('59).

Albino rats were raised in wire-bottom cages and fed purified diets and water ad libitum. The rats were assigned from the Food and Drug Research Laboratory colony, and the composition of the diets was that described previously (Brin et al., '60), except that in this case, pyridoxine was omitted from the basic formula when fed to the deficient groups.

Blood was drawn by cardiac puncture into heparinized syringes. After centrifugation, the plasma was removed for analysis of the alanine and aspartic transaminases. (Obtaining the blood sample by decapitation resulted in highly variable values, probably due to release of enzyme from the excised tissues.)

Enzyme incubations were performed at 37°. A colorimetric technique was used in which the pyruvate formed in the reaction was isolated from the acid-treated incubation mixture as the phenylhydrazone. The color was developed with alcoholic KOH, and read in the photoelectric

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[1] This study was initiated under Contract no. DA-49-007-MD-862 of the Surgeon General's Office to the Food and Drug Research Laboratories, Inc., and extended at Syracuse with the support of the Research Foundation of the State of New York and the Williams-Waterman Fund.

[2] Brin, M., and R. E. Olson 1951 Effect of pyridoxine deficiency upon respiration and transaminase activity of cardiac muscle in ducklings. Federation Proc., *10:* 166 (abstract).

[3] See footnote 2.

[4] Brin, M., and M. Tai 1958 The effects of pyridoxine deficiency on serum transaminases. Federation Proc., *17:* 472 (abstract).

colorimeter at 490 mμ (Cabaud et al., '56; Wroblewski and Cabaud, '57). This method proved more suitable than our previously described assays,[5] (Brin et al., '54).

Where added to the assay, B_6P was incubated with the plasma for 20 minutes before the substrate was added. The units in which the data are expressed are equivalent to the production of micrograms of pyruvate per milliliter of plasma per hour.

RESULTS

The growth response of rats fed a pyridoxine-deficient diet was typical (fig. 1). In this deficiency, growth was markedly depressed almost immediately—certainly by the first week. Physical signs of deficiency, tremor, etc., however, were not usually evident until receiving the deficient diet for three weeks.

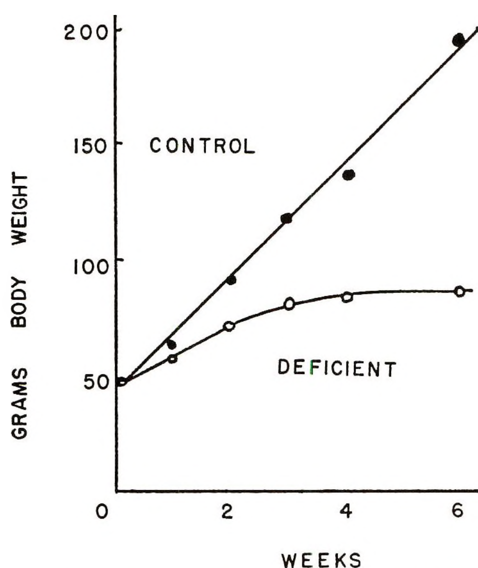

Fig. 1 Rat growth with a pyridoxine-deficient diet.

The effects of pyridoxine deficiency on two transaminases in plasma from young rats are shown in table 1. The rats started to receive the test diets when they weighed 49 gm, and were withdrawn from the feeding program between the second and third weeks, for assay. It was evident that in the plasma of control rats the aspartic enzyme was more active than the alanine enzyme. The addition of B_6P to each control plasma did not stimulate the enzyme activity to higher levels. The activity of the alanine transaminase in the deficient group was depressed approximately 85%. The addition of B_6P to these plasmas increased the enzyme activity appreciably, although not completely to normal values. The activity of the aspartic enzyme in the deficient plasmas was depressed about 63%. Supplementation with B_6P did not stimulate the aspartic enzyme.

With the demonstration that B_6P stimulated the alanine enzyme *in vitro*, though not the aspartic enzyme, it was of interest to determine what effect might be elicited by repletion of the intact animal with the limiting vitamin. Observations are presented in table 2. Groups of deficient rats were fed the control diet, on the one hand, or continued to receive the deficient diet supplemented with two injections of pyridoxine·HCl administered intraperitoneally at two-day intervals and at a level of of 2 mg per injection. It was evident, that after feeding the deficient diet for an initial period of two to 4 weeks, transfer to the control diet restored the activity of the alanine

[5] See footnote 2.

TABLE 1

Effect of pyridoxine deficiency on plasma transaminases in young rats[1]

Group	No. rats	Alanine enzyme			Aspartic enzyme		
		No addition	$+B_6P$[2]	Depression[3]	No addition	$+B_6P$[2]	Depression[3]
		units		%	*units*		%
Control	13	378 ± 74[4]	378 ± 106		831 ± 211[4]	865 ± 256	
				85			63
Deficient	6	55 ± 24[4]	109 ± 28		333 ± 87[4]	333 ± 87	

[1] These rats were on test two to 4 weeks at time of assay. Data are presented as mean ± S.E.
[2] Before adding substrate, 50 μg of B_6P were incubated with plasma.
[3] Percentage of depression calculated as $\frac{\text{control} - \text{deficient}}{\text{control}} \times 100$.
[4] $P < 0.01$.

TABLE 2

Effect on serum transaminases of repletion of deficient rats with pyridoxine in vivo[1]

Group	Treatment	No. rats	Alanine		Aspartic	
			No addition	+B_6P[2]	No addition	+B_6P[2]
			units		*units*	
Control	—	13	378 ± 74		831 ± 211	
Deficient, 2–4 weeks	none	6	55 ± 24		361 ± 21	
Deficient, 2–4 weeks	control diet, 4 days	6	373 ± 164	374 ± 134	611 ± 131	556 ± 114
Deficient, 2–4 weeks	injection B_6 I.P. 2 × 2 mg	12	296 ± 107	265 ± 101	733 ± 194	722 ± 206

[1] These rats were on test two to 4 weeks at time of assay. Data are presented as mean ± S.E.
[2] Before adding substrate, 50 μg of B_6P were incubated with plasma.

enzyme to normal and the aspartic enzyme to 75% of normal. Supplementation of deficient rats by injection of pyridoxine·HCl also elicited positive responses. The alanine enzyme was stimulated to 80% of the control values, and the aspartic enzyme to 90% of normal, for these rats in only 4 days. Adding B_6P *in vitro* had no further stimulating effect on either enzyme in the plasmas obtained from rats which were treated either by feeding pyridoxine or by injection of the vitamin.

It was of further interest to determine whether serum transaminases would be depressed in adult, slow-growing rats, as readily as in young, more rapidly growing animals. Accordingly, control and deficient diets were administered to rats weighing in excess of 225 gm, and after two weeks, the rats were subjected to transaminase assays. The results are presented in table 3. It was evident that plasma transaminases were readily depressed in pyridoxine-deficient adult rats as rapidly in younger animals though not to the same extent. The alanine enzyme was once again affected more markedly than the aspartic enzyme. The addition of B_6P *in vitro* had no marked stimulating effect on the activity of either enzyme in the plasmas from deficient or control adult rats.

DISCUSSION

It is of nutritional interest that a plasma enzyme, readily available from a subject without detriment, is sensitive to a specific vitamin deficiency. In young animals both the alanine and the aspartic enzymes were depressed to less than half-normal after receiving a pyridoxine-deficient diet for approximately three weeks. The observation that the addition of B_6P to the deficient plasma samples resulted in enhanced activity of the alanine enzyme, demonstrated that the transaminase effect was due to inadequate coenzyme relative to available apoenzyme in the plasma; in other words, apoenzyme was observed in excess of available coenzyme. The fact that B_6P did not result in complete restoration indicated that in pyridoxine deficiency, the availability of apoenzyme was also probably curtailed. It would appear that where the addition of B_6P had no positive effect, all of the available apoenzyme in the plasma sample was saturated with the coenzyme.

Despite the slower growth rate and lower vitamin requirement of adult animals, the heavier rats were also depleted of transaminase activity in as little as two weeks on test.

The differential effect seen between the extent of depression of the alanine and the aspartic enzyme, both in young and in adult animals, lends further support to the belief that these enzymes are two different proteins. By virtue of the fact that the alanine enzyme in tissues too is affected more readily under many conditions of physiological stress[6] (Awapara, '53; Brin et al., '54), it may be concluded that the alanine enzyme may have a lower affinity

[6] Brin, M., and R. W. McKee 1952 Effects of x-radiation on tissue transaminases. Federation Proc., *11*: 190 (abstract).

TABLE 3

Effect of pyridoxine deficiency on plasma transaminases in heavy rats[1]

Group	Alanine enzyme				Aspartic enzyme			
	No. rats	No addition	$+B_6P$[2]	Depression[3]	No. rats	No addition	$+B_6P$[2]	Depression[3]
		units	*units*	%		*units*	*units*	%
Control	6	379.5 ± 23.4	351.2 ± 30.4		10	564.7 ± 39.2	609.4 ± 35.7	
				60				22
Deficient	13	153.6 ± 28.7	163.1 ± 12.5		22	440.3 ± 18.2	451 ± 30.4	

[1] These rats were on test two to 4 weeks at time of assay. Data are presented as mean ± S.E.

[2] Before adding substrate, 50 µg of B_6P were incubated with plasma.

[3] Percentage of depression calculated as $\frac{\text{control} - \text{deficient}}{\text{control}} \times 100$.

for the coenzyme, or that the rate of turnover of the apoenzyme-coenzyme may be greater for the alanine than for the aspartic enzyme. In any event it would appear that the alanine enzyme may be the more sensitive test for pyridoxine status.

Aside from the nutritional aspects of plasma transaminases described in this paper, it is of additional interest that these enzymes were depressed in the light of the current view that they do not originate in blood but are derived from peripheral tissues (LaDue et al., '54; Wolfson et al., '58). The depressed plasma enzymes lend support to the argument that frank necrosis is not a necessary condition for the release of these proteins into the circulation. Furthermore, at least for the alanine enzyme, it would appear that tissues are permeable to apotransaminase as well as to the intact enzyme.

The studies presented in this report are an extension of efforts from our laboratories to obtain enzyme parameters in blood which reflect specific vitamin deficiencies[7,8] (Brin et al., '58; Surgeon General, '58, '59; Wolfe et al., '58). The plasma transaminase technique described in this paper has been used successfully as an evaluation tool for pyridoxine deficiency in studies on the effects of feeding to rats foods which were sterilized by exposure to high levels of X-irradiation. The data are reported elsewhere (Surgeon General, '58, '59).

SUMMARY

Rats, upon being fed diets deficient in pyridoxine for periods of two to 4 weeks, showed markedly depressed activity of two plasma transaminases. In young rats, the alanine enzyme was affected more markedly (85%) than the aspartic enzyme (63%). In adult rats, too, the alanine enzyme was more markedly depressed (60%) than the aspartic enzyme (22%) although the total effect was not as great as in the younger animals. Partial restoration of serum alanine transaminase enzyme activity was elicited by incubating the deficient serum with pyridoxal phos-

[7] Brin, M., S. S. Shohet and C. S. Davidson 1956 Effect of thiamine deficiency on mammalian erythrocyte metabolism. Federation Proc., *15*: 224 (abstract).

[8] See footnote 4.

phate before adding the assay substrate. The activities of both the alanine and the aspartic enzymes of serum obtained from deficient rats were rapidly restored by (a) injecting the deficient rats with pyridoxine·HCl, or (b) by feeding the control diet to the deficient rats. The application of the use of these enzymes to the evaluation of the availability of vitamin B_6 in foods which were preserved by ionizing radiation was indicated.

ACKNOWLEDGMENTS

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Invitations for Nominations for 1961 American Institute of Nutrition Awards

Nominations are requested for the 1961 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed and (2) a statement as convincing as possible as to the basis for the nomination, stating the eligibility of the candidate (this may include the pertinent bibliography of the most appropriate and significant recent papers on which the nomination is based, but such bibliography is not necessary unless later requested by the Nominating Committee). Reprints are not required, nor are seconding statements. *Five copies of all documents* must be sent to the chairman of the appropriate Nominating Committee *before December 1, 1960,* to be considered for the 1961 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award.[1] A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made at the annual dinner at the annual meeting.

[1] Including recipients of the former Mead-Johnson award. These are listed at the end of this notice.

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The Borden Award in Nutrition, consisting of $1,000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

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1947—L. A. Maynard
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1950—H. C. Sherman
1951—P. György
1952—M. Kleiber
1953—H. H. Williams
1954—Agnes Fay Morgan and A. H. Smith
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1957—no award
1958—L. D. Wright
1959—H. Steenbock
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G. V. Mann, *Chairman*
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1961 Osborne and Mendel Award

The Osborne and Mendel Award of $1,000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

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1941—R. J. Williams
1942—G. R. Cowgill
1943—V. du Vigneaud
1944—A. G. Hogan
1945—D. W. Woolley
1946—E. E. Snell
1947—W. J. Darby, P. L. Day, and E. L. R. Stokstad
1948—F. Lipmann
1949—Mary S. Shorb and K. A. Folkers
1950—W. B. Castle
1951—no award
1952—H. E. Sauberlich

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Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

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ERRATA

The publishers regret the occurrence of typographical errors in issues 1, 2, and 3 of volume 71. The corrected passages are printed below for the purpose of cutting and pasting over the incorrect printing.

PRIVETT, O. S., F. J. PUSCH, R. T. HOLMAN AND W. O. LUNDBERG 1960 Essential fatty acid properties of tuna, herring and menhaden oils. J. Nutrition, *71:* 66.

Symbols and group numbers in figures 1 and 2 should be corrected as follows:

Page 67 — Fig. 1		Page 68 — Fig. 2	
⊗———⊗	GROUP 1	△———△	GROUP 1
△———△	GROUP 2	□———□	GROUP 2
□———□	GROUP 3	●———●	GROUP 3
○———○	GROUP 4	⊗———⊗	GROUP 4
●———●	GROUP 5	○———○	GROUP 5

Fig. 2 Average dermal scores of rats in experiment 2 (part 2). Groups 1, 2, 3, and 4 received basal diet plus 10% menhaden, tuna, herring, and corn oil respectively; group 5 received only basal fat-free diet.

BARNES, R. H., E. KWONG, K. DELANY AND G. FIALA 1960 The mechanism of the thiamine-sparing effect of penicillin in rats. J. Nutrition, *71:* 149.

The graphs in figures 1 and 2 were interchanged. That in figure 2 should accompany the legend in figure 1 and vice versa as corrected below.

Corrected

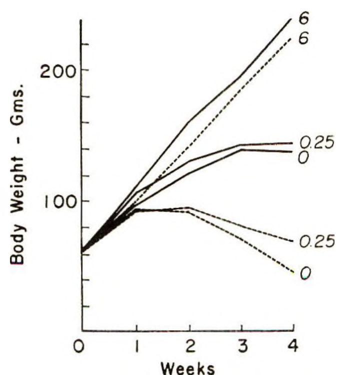

Fig. 1 Growth of rats receiving three levels of thiamine in a diet without penicillin. Solid lines, coprophagy permitted, and broken lines, coprophagy prevented. Numbers at the right are milligrams of thiamine hydrochloride per kilogram of diet.

Corrected

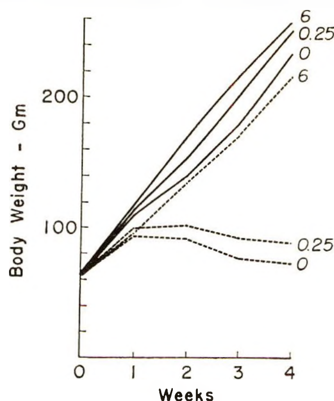

Fig. 2 Growth of rats receiving three levels of thiamine in a diet with penicillin. Solid lines, coprophagy permitted, and broken lines, coprophagy prevented. Numbers at the right are milligrams of thiamine hydrochloride per kilogram of diet.

Errata continued on next page