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Functional Pancreatic Damage Produced by Ethionine, and its Relation to Methionine Deficiency[1]

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The pancreas is adapted for rapid synthesis of large quantities of protein. It would not be unexpected, therefore, if deficiences of essential amino acids seriously impaired its functional activity. One such amino acid, methionine, appears to be especially important for maintaining pancreatic integrity. Wachstein and Meisel ('54) showed that 1% of methionine protected the rat pancreas from pathological damage induced by feeding a nonprotein diet. Similar effects have been reported for rats and dogs fed methionine-deficient diets (Sós and Kemény, '56). By means of S^{35}-labeled methionine, it was shown that methionine localized in the pancreas during active secretion (Wheeler et al., '49; Friedberg et al., '48). Apparently the uptake by this organ is rapid, since significant amounts were incorporated after only 16 minutes (Edwards et al., '60).[2]

Considerable evidence shows that ethionine, the thioethyl analogue of methionine, produced severe pathological lesions in the pancreas when fed or injected (Farber and Popper, '50; Goldberg et al., '50; Wachstein and Meisel, '51; Popper et al., '52). The lesions apparently result in depressed exocrine function (De Almeida and Grossman, '52; Kroboth and Hallenbeck, '54; Lin and Grossman, '54) since the administration of ethionine reduced the output of digestive enzymes in pancreas-fistulated dogs. Simultaneous administration of methionine usually prevented the pathological as well as the functional changes. Therefore, ethionine is generally considered to promote a functional methionine deficiency (Farber et al., '50; Sidransky and Farber, '58; Stein et al., '60). Much of the evidence suggests that the compound interferes with methionine metabolism and hence with normal protein synthesis (Simpson et al., '50; Lee and Williams, '52).

A report by Sidransky and Farber ('56) that ethionine increased pancreatic amylase and protease synthesis in the rat 24 hours after injection seemed to contradict the belief that protein synthesis was inhibited by the amino acid antagonist. Therefore, it became the purpose of these experiments to investigate further the influence of ethionine on pancreatic enzyme secretion in the rat. In addition, some of the effects of ethionine were compared with those produced by a specific methionine deficiency.

EXPERIMENTAL

Animals and diets. Female, Long-Evans rats weighing 165 to 200 gm were used throughout the experiments. They were conditioned in individual cages for two or three days before an experiment, and then fed the experimental diets. Composition of the basal diets and of the amino acid mix is shown in table 1. Supplements of ethionine or methionine were added at the expense of sucrose.

Procedure. Rats were allowed free access to their respective diets for 5 days during which a daily record of food consumption and weight gain was kept. On the 6th day, following an overnight fast, the animals were tube-fed either 4 to 5 gm of diet

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[2] Edwards, C. H., E. L. Gadsden and G. A. Edwards 1960 Early uptake of DL-methionine 2-C^{14} and L-methionine CH_3^{14} by rat tissues. Federation Proc., *19*: 42 (abstract).

TABLE 1

Basal diets and amino acid mix composition

Ingredient	Basal diets		Amino acid mix[1]	
	1	2		%
Casein	18	—	L-lysine	1.78
			L-arginine	0.91
Sucrose	57	46.5	L-tryptophan	0.27
			DL-phenylalanine	1.04
Amino acid mix (minus methionine)	—	27.9	DL-leucine	3.64
			DL-isoleucine	2.38
			DL-valine	2.80
Fortified oil[2]	1	1	L-histidine	0.68
USP XIV salts	4	4	DL-methionine	(0.60)
			DL-threonine	1.62
Cottonseed oil	9	9	DL-serine	2.26
			glycine	0.38
Celluflour[3]	10	10	DL-tyrosine	1.13
			L-cystine	0.27
Vitamin mix[4]	1	1	L-proline	2.27
			L-aspartic acid	1.17
			L-glutamic acid	4.22
DL-Methionine	—	0.6	DL-alanine	1.12
	100	100		27.94

[1] Amino acid mix was compounded according to summary tables in Block and Weiss ('56) to approximate the L-amino acids in 18% casein. An additional 0.2% of L-cystine was added to the mix. Since the D-forms of leucine, isoleucine, valine and threonine are not completely utilized by the rat, the concentration of these DL-amino acids was doubled.

[2] Fortified oil provided per 100 gm of diet: vitamin A, 1700 I.U.; vitamin D, 100 I.U.; α-tocopheryl acetate, 6.66 mg; menadione, 0.5 mg.

[3] CelluFlour, Chicago Dietetic Supply House, Chicago.

[4] Vitamin mix provided as mg per 100 gm of diet: thiamine, 0.2; riboflavin, 0.3; pyridoxine, 0.25; Ca pantothenate, 2.0; inositol, 10.0; biotin, 0.01; folic acid, 0.02; niacinamide, 1.0; vitamin B_{12}, 0.02; choline chloride, 50.0.

or diet plus a suspension of 160 mg of a concentrate of soybean trypsin inhibitor (SBTI).[3] Similar material had previously been shown to stimulate strongly the discharge of pancreatic enzymes when fed orally (Lyman and Lepkovsky, '57). One or two hours after feeding, rats were killed, and the pancreas was removed, freed of visible fat under a dissecting microscope and lyophilized. Contents from the small intestine were also removed, frozen and lyophilized. In some of the experiments, stomach contents were removed and dried in an oven for dry-weight determination. The dry pancreatic and intestinal material was ground, and aliquots were homogenized in water and analyzed for lipase, protease,[4] and amylase activities by methods previously described (Lyman and Lepkov-

[3] The SBTI concentrate was prepared from whole, uncooked soybeans by homogenizing 100 gm in one liter of water, adjusting the pH to 4.2 and extracting overnight. The supernatant was obtained by centrifuging. To this clear solution was added ammonium sulfate (30 gm/100 ml). The precipitated protein was dissolved in water, and the ammonium sulfate precipitation was repeated once more. The precipitate was again dissolved in water and dialyzed 16 hours against tap water at room temperature. The dialyzed solution was lyophilized and stored as a dried powder. Its trypsin inhibiting activity was 25 to 30 times that of the original soybeans.

[4] Activation of trypsinogen in pancreases was accomplished by incubation for 20 minutes with an 0.5% suspension of Viodenum (Viobin Corporation, Cleveland, Ohio). The term protease is used to indicate all forms of proteolytic activity produced by carboxypeptidase, aminopeptidase, trypsin and chymotrypsin. Incubation of the pancreatic tissue with hemoglobin without this preliminary activation showed no activity.

sky, '57). Nitrogen was determined by an all-glass, semimicro Kjeldahl apparatus after digestion with sulfuric acid (copper sulfate as catalyst).

RESULTS

Effect of ethionine in a casein diet. An 18% casein diet providing approximately 0.6% of methionine was supplemented either with 0.2% of DL-ethionine or with ethionine and 0.4% of DL-methionine, and fed as described. Rats were killed at one- and two-hour intervals after the tube feeding, and the pancreases and intestinal contents were analyzed for lipase, protease, and amylase activity.

Figure 1 shows the results of these analyses. With the basal diet, only a slight pancreatic stimulation was produced as indicated by the relatively high pancreatic and low intestinal activities of the three enzymes. However, the pancreatic enzyme activities in the rats receiving ethionine with or without additional methionine were consistently higher than those of the controls. This was especially true for amylase activity. Intestinal enzymes remained lowest for rats fed ethionine with

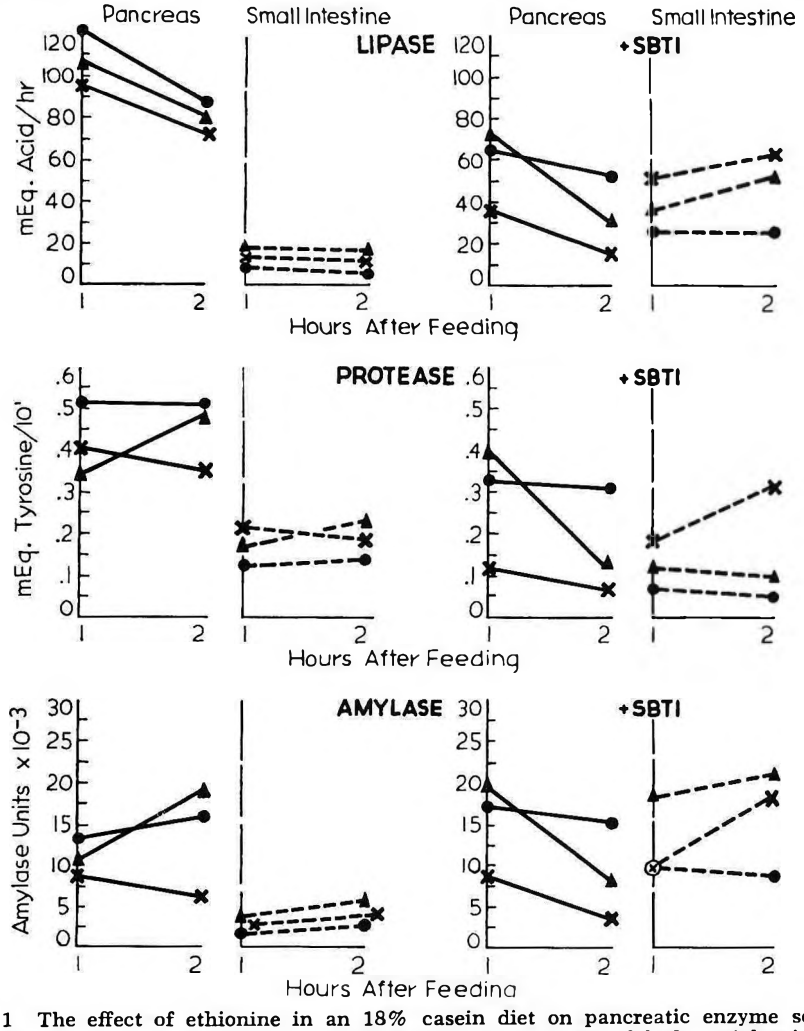

Fig. 1 The effect of ethionine in an 18% casein diet on pancreatic enzyme secretion. Pancreatic enzymes are expressed as activity units per 100 gm of body weight; intestinal activity as total activity of contents. Each point represents the average from two individual rats. X—X, 18% casein basal; ●—●, Basal + 0.2% DL-ethionine; △—△, Basal + 0.2% DL-ethionine + 0.4% DL-methionine.

TABLE 2

Pancreatic enzyme secretion in rats fed amino acid diets supplemented with ethionine and methionine

Diet	No. rats	Diet alone					
		Pancreas activity/100 gm body weight			Total intestinal activity		
		Lipase	Protease	Amylase	Lipase	Protease	Amylase
		mEq acid per hour	*mEq tyrosine/10'*	*amylase units* $\times 10^{-3}$	*mEq acid per hour*	*mEq tyrosine/10'*	*amylase units* $\times 10^{-3}$
Basal[1] + 0.6% methionine (control)	3	77.6± 4.2[2]	0.27±0.01	8.2 —	2.8±0.6	0.18±0.05	2.7±0.8
Basal + 0.1% ethionine	3	61.5±13.7	0.46±0.02	15.3±2.6	4.1±2.2	0.05±0.02	2.3±0.8
Basal + 0.1% ethionine + 0.2% methionine	3	75.0± 9.0	0.33±0.07	10.3±3.2	7.6±2.5	0.05±0.01	1.6±0.3
Basal + 0.1% ethionine + 0.4% methionine	2	79.8 —	0.54 —	21.1 —	7.1 —	0.08 —	1.7 —
Basal + 0.1% ethionine + 0.8% methionine	2	66.0 —	0.63 —	20.1 —	7.1 —	0.20 —	4.9 —
		Diet + 160 mg SBTI					
Basal + 0.6% methionine (control)	4	33.0± 6.1	0.12±0.02	5.0±0.1	42.5±3.9	0.15±0.03	16.2±2.4
Basal + 0.1% ethionine	5	56.9± 2.9	0.31±0.03	14.1±1.9	7.3±3.1	0.02 —	4.7±1.8
Basal + 0.1% ethionine + 0.2% methionine	3	55.4± 5.0	0.31±0.03	14.4±1.9	5.5±3.2	0.01 —	4.1±0.9
Basal + 0.1% ethionine + 0.4% methionine	3	34.3± 8.9	0.23±0.04	8.2±1.8	16.7±3.2	0.05±0.02	5.8±1.6
Basal + 0.1% ethionine + 0.8% methionine	3	17.1± 6.3	0.12±0.03	5.5±1.0	21.3±3.0	0.11±0.06	9.0±2.4

[1] Basal diet indicates amino acid diet devoid of methionine.
[2] Values represent standard error of the mean.

no supplemental methionine. With additional methionine in the diet, the enzyme activities in this group seemed to be the highest. These relatively slight changes were more obvious in those animals treated with SBTI. Whereas discharge of pancreatic enzymes was indicated in the controls by depressed pancreatic enzyme activities, those rats fed ethionine with no methionine exhibited very little loss of pancreatic activity. Also, the enzyme depletion produced in the control rats was accompanied by large increases in intestinal enzyme activities. No such increase in the intestine took place in the ethionine-fed group. However, additional methionine in the ethionine diet promoted nearly normal pancreatic response to the SBTI. The exception seemed to be protease which failed to increase in the intestine as lipase and amylase did. Possibly the intestinal activity of this enzyme may have less meaning than that of the others since SBTI inactivates some undetermined portion of the secreted trypsin. When protease secretion is stimulated by SBTI, however, a definite rise in intestinal activity is usually produced.

The results indicated that dietary ethionine interfered with pancreatic function by blocking enzyme secretion into the digestive tract. The completeness of this secretory inhibition was unexpected since the casein in the diet provided approximately 0.6% of methionine, yet an additional 0.4% of methionine was needed to counteract the effects of the analogue.

Effect of ethionine in amino acid diets. The possibility that the methionine in the casein was unavailable to the ethionine-fed animal was explored by means of purified amino acid diets compounded in such a way so as to provide L-amino acids or their equivalents in the proportions found in casein. All diets except the control contained 0.1% of DL-ethionine. Methionine was added to the otherwise methionine-deficient diet at zero, 0.2, 0.4 and 0.8% of the ration. In this way all the methionine was free and presumably equally available for absorption. The experiments were carried out as before except that only the two-hour postfeeding period was considered. As shown in table 2, lipase activity in those pancreases stimulated by diet alone remained high and was similar for all groups, whereas protease and amylase activities exceeded the controls in all ethionine-fed groups. The highest values for these enzymes were attained at the two highest methionine supplementations. Intestinal lipase seemed to be slightly in-

TABLE 3

Percentage of food in stomach, quantity of intestinal contents, food intake and growth response of ethionine- and methionine-supplemented rats

Diet	No. rats	Food in stomach after 2 hours	Total weight of intestinal contents	Av. food eaten/rat during 5 days	Av. 5-day weight change/rat
		%	mg[1]	gm[1]	gm
Basal+0.6% methionine (control)	5	60.6±1.5[2]	369±10	65±10	5±2
Basal+0.1% ethionine	9	57.9±4.6	320±43	24±3	−20±3
Basal+0.1% ethionine +0.2% methionine	6	63.1±4.7	420±22	27±5	−11±2
Basal+0.1% ethionine +0.4% methionine	5	66.4±4.0	203±52	39±3	− 9±4
Basal+0.1% ethionine +0.8% methionine	5	66.5±3.2	337±59	36±5	− 9±3
Casein basal (control)	9	—	—	62±3	10±2
Casein basal+0.2% ethionine	9	—	—	28±3	−16±4
Casein basal+0.2% ethionine +0.4% methionine	9	—	—	37±4	−10±3

[1] Values represent weight of dry solids.
[2] Standard error of the mean.

creased in the ethionine groups, but the difference was probably not significant. Protease activity remained especially low in the ethionine-fed rats and approached normal only with 0.8% of methionine in the diet. When pancreas secretion was forced by SBTI, control rats showed the typical enzyme depletion, accompanied by increased intestinal enzyme activity. However, the animals fed ethionine or ethionine and 0.2% of methionine, did not secrete in response to the SBTI as evidenced by maintenance of high pancreatic enzyme activities and low intestinal activities. With 0.4% of methionine in the diet, the ethionine-fed rats began to show some improvement in enzyme secretion. While at 0.8% of methionine, the pancreas secreted nearly all of its enzymes and intestinal enzyme activity was greatly increased. The results therefore confirmed those obtained with ethionine and intact protein, and showed that a considerable excess of methionine was required to prevent the inhibition of pancreatic enzyme secretion.

Inasmuch as some of the conclusions reached in this study were based on the results of intestinal enzyme activities, the rate of removal of food from the stomach and the total quantity of intestinal contents were compared in ethionine-fed rats and controls. A low intestinal enzyme activity might be anticipated if ethionine inhibited stomach emptying, thereby reducing subsequent amounts of intestinal contents. Table 3 shows that the percentage of food emptied from the stomachs of the control animals did not differ appreciably from the amount removed from stomachs of the ethionine-fed animals. Also, the quantities of intestinal contents were comparable for all groups, and thus could not account for the differences observed. Table 3 shows also that, regardless of the level of supplementation with methionine, all rats fed ethionine had reduced food intakes with concomitant weight loss. This weight loss was not considered a major factor influencing the enzyme results since some animals failed to secrete whereas others, which had eaten only slightly more and lost nearly the same weight, did respond to stimulation.

It has been reported that ethionine inhibits transmethylation of methionine to

TABLE 4

Pancreatic enzyme secretion in rats fed amino acid diets and ethionine supplemented with choline

Diet[1]	No. rats	Pancreas activity/100 gm body weight			Total intestinal activity		
		Lipase	Protease	Amylase	Lipase	Protease	Amylase
		mEq acid per hour	*mEq tyrosine/10'*	*amylase units* $\times 10^{-3}$	*mEq acid per hour*	*mEq tyrosine/10'*	*amylase units* $\times 10^{-3}$
Basal + 0.1% ethionine + 0.39% choline	3	63.5 ± 6.6[2]	0.47 ± 0.03	13.0 ± 1.8	2.2 ± 1.3	0.02 ± 0.01	2.1 ± 0.6
Basal + 0.1% ethionine + 0.39% choline + 0.2% methionine	3	62.5 ± 4.2	0.44 ± 0.01	13.4 ± 1.9	2.5 ± 0.8	0.02	3.2 ± 0.8
Basal + 0.1% ethionine + 0.39% choline + 0.8% methionine	3	34.0 ± 2.7	0.24 ± 0.02	9.9 ± 1.0	27.6 ± 5.8	0.14 ± 0.03	13.5 ± 3.1

[1] Two hours prior to autopsy all rats were fed 4 to 5 gm of their respective diets + 160 mg of SBTI.
[2] Standard error of the mean.

choline (Simmonds et al., '50). The diets in these experiments had been compounded to contain 0.05% of choline chloride. Under the conditions of the experiment, it seemed likely that a choline deficiency could have been induced. Therefore, an experiment was performed using the diets previously described, but containing 0.39% of choline (0.45% choline chloride). The results shown in table 4 are nearly identical with those obtained earlier, indicating that additional choline had no effect upon the secretory response of the pancreas.

Others who have studied the relationship between pancreas function and ethionine have generally used parasympathomimetic drugs such as carbamylcholine, urecholine or methacholine as pancreas stimulants. In the present studies a dietary agent, soybean trypsin inhibitor, was used as a pancreas stimulant. Very little is known about its mechanism of action, but apparently it does not act entirely through parasympathetic pathways (Lyman, '59).[5] To determine whether neural stimulation would give the same results, animals fed ethionine-supplemented amino acid diets for 5 days were then injected with methacholine and secretin and their residual pancreatic enzyme activities compared with controls. It had been our experience that methacholine alone did not produce significant enzyme changes in the short period of time observed, and that only the combination of secretin and methacholine was effective. Table 5 shows that ethionine impaired enzyme discharge induced by methacholine as it did with the SBTI.

Effect of ethionine and amino acid diets on renewal of pancreatic enzyme activity. The toxic effects of ethionine have been interpreted by many investigators to result from its interference with the normal synthesis of protein. In view of the report that ethionine may have stimulated enzyme synthesis in the rat pancreas some time after injection (Sidransky and Farber, '56), this possibility was investigated under our experimental conditions. Table 6 presents pancreatic enzyme activities obtained from animals fed their respective diets plus SBTI, then killed following a 22-hour fast. The importance of methionine for restoring pancreatic enzyme activity is evident from the results. With 0.2% of methionine in the diet, ethionine-fed rats were unable to maintain the pancreatic enzyme activities present two hours after stimulation. This loss of activity was also reflected in the reduced nitrogen of the pancreases, suggesting that renewal of enzyme protein may have been impaired. As dietary methionine was increased, pancreatic nitrogen increased as did lipase and amylase. Although protease activity seemed to be relatively uninfluenced by methionine, amylase activity increased to

[5] Lyman, R. L. 1959 Investigations on the mechanism of the pancreas stimulating action of unheated soybean antitrypsin in the rat. Federation Proc., *18:* 2103 (abstract).

TABLE 5

Stimulation of pancreatic secretion by methacholine and secretin 20 minutes after injection, and the effect of dietary ethionine

Diet and treatment[1]	No. rats	Pancreas activity/100 gm body weight		
		Lipase	Protease	Amylase
		mEq acid/hour	*mEq tyrosine/10'*	*amylase units* $\times 10^{-3}$
Basal + 0.6% methionine (control) (100 γ methacholine-Cl + 2 mg secretin)[2]	3	32.4 ± 4.2[3]	0.17 ± 0.03	4.8 ± 0.9
Basal + 0.1% ethionine (100 γ methacholine-Cl + 2 mg secretin)	5	51.5 ± 6.5	0.34 ± 0.02	11.6 ± 1.9

[1] Rats were used following a 22-hour fast. One milligram of ethyl carbamate per gram of body weight was injected subcutaneously. This amount kept the rats under light anesthesia during infusion of the secretin.

[2] Methacholine (acetyl-β-methylcholine) injected subcutaneously; secretin injected into femoral vein. Secretin was generously supplied by Dr. M. H. F. Friedman, Jefferson Medical College and Dr. H. J. Henry of Lilly Research Laboratories.

[3] Standard error of the mean.

TABLE 6

Renewal of pancreatic enzymes and nitrogen 22 hours after stimulation by diet and SBTI

Diet	No. rats	Pancreas activity/100 gm body weight			
		Lipase	Protease	Amylase	N_2/pancreas/100 gm body weight
		mEq acid/hour	*mEq tyrosine/10'*	*amylase units* $\times 10^{-3}$	*mg*
Basal + 0.6% methionine (control)	5	66.8 ± 3.5[1]	0.27 ± 0.04	6.6 ± 0.8	13.2 ± 0.7
Basal + 0.1% ethionine	3	30.7 ± 3.2	0.21 ± 0.03	5.0 ± 1.2	7.35 ± 0.4
Basal + 0.1% ethionine + 0.2% methionine	2	26.1 —	0.20 —	4.9 —	8.34 —
Basal + 0.1% ethionine + 0.4% methionine	3	39.5 ± 5.4	0.28 ± 0.04	7.1 ± 1.5	10.5 ± 0.6
Basal + 0.1% ethionine + 0.8% methionine	3	52.6 ± 1.5	0.29 ± 0.02	14.7 ± 2.3	12.9 ± 0.5

[1] Standard error of the mean.

twice the control value when the highest concentration of the amino acid was fed. Although a considerable excess of methionine, relative to the ethionine, was required to restore pancreatic nitrogen, lipase and protease to nearly normal, it did not prevent the abnormal increase in pancreatic amylase.

Effect of feeding a methionine-deficient diet. Because most of the effects produced by ethionine were improved when sufficient methionine was fed simultaneously the effect of a specific methionine deficiency on pancreatic secretion was next investigated.

Two experiments were performed, using the basal amino acid diet devoid of methionine. In the initial experiment, rats were allowed to eat ad libitum until average weight loss was similar to that produced in ethionine-fed rats. This required about 12 days. The animals were then fed the diet and SBTI, and were sacrificed after two hours. Table 7 shows that no real difference existed between deficient and control groups in either the pancreatic or intestinal enyme levels. On the assumption that a complete deficiency had not been produced, a force feeding technique was used to accelerate and intensify the deficiency state. After 12 days of feeding the complete and deficient diets, only the deficient animals exhibited porphyrin staining about the paws and nose, weight loss and some muscular weakness. No diarrhea or other gastrointestinal disturbances were noticed. Stimulation of pancreatic secretion in these animals resulted in a severe depletion of pancreas enzymes in both control and deficient animals. However, in the deficient animals, intestinal enzyme activity was only about one-half the activity produced by control rats. Motility of food from the stomach was similar for both groups, so could not account for this difference. Therefore, a specific deficiency of methionine did not appear to inhibit the secretory function of the pancreas, but reduced the amount of activity secreted into the small intestine.

DISCUSSION

Observations made by others, on dogs and under different conditions, have also suggested that ethionine may interfere with pancreatic secretory response. De Almeida and Grossman ('52) demonstrated, in pancreatic-fistulated dogs, that large doses of ethionine injected over an interval of a few days produced depressed secretion of pancreatic enzymes when stimulated by secretin and urecholine. Secretin stimulation alone produced no difference between ethionine and control animals. It was concluded that the decreased enzyme output was primarily the result of protein synthesis inhibition. Kroboth and Hallenbeck ('54) fed ethionine in small capsules to dogs and reported that serum amylase activity increased, while amylase of the pancreatic juice decreased. Kalser and Grossman ('54) ob-

TABLE 7

Pancreatic enzyme activity in methionine-deficient rats

Diet and treatment[1]	No. rats	Pancreas activity/100 gm body weight			Total intestinal activity			Food eaten over 12 days	12-Day weight change
		Lipase	Protease	Amylase	Lipase	Protease	Amylase		
		mEq acid/hour	*mEq tyrosine/10'*	*amylase units* $\times 10^{-3}$	*mEq acid/hour*	*mEq tyrosine/10'*	*amylase units* $\times 10^{-3}$	*gm*	*gm*
Ad libitum									
Basal, no methionine fed for 12 days	4	27.8 ± 4.7[2]	0.15 ± 0.03	5.9 ± 0.39	29.6 ± 7.0	0.12 ± 0.04	11.3 ± 1.6	90 ± 4	−25 ± 2
Basal + 0.6% methionine fed for 12 days	3	18.0 ± 4.4	0.11 ± 0.03	4.7 ± 1.2	29.4 ± 2.3	0.11 ± 0.01	10.7 ± 2.1	162 ± 2	10 ± 4
								% food in stomach	12-Day weight change
Force-fed[3]									
Basal, no methionine fed for 12 days	5	2.7 ± 1.0	0.02 ± 0.002	1.1 ± 0.2	15.3 ± 3.5	0.09 ± 0.02	4.3 ± 0.9	64.8 ± 4.6	−17 ± 1
Basal + 0.6% methionine fed for 12 days	4	4.8 ± 1.0	0.04 ± 0.006	2.6 ± 0.4	47.6 ± 2.6	0.17 ± 0.01	10.6 ± 0.7	68.5 ± 2.7	3 ± 1

[1] All rats fed diet + 160 mg SBTI two hours prior to autopsy.
[2] Standard error of the mean.
[3] Force-fed rats received 11 gm diet daily.

served that ethionine-injected dogs showed increased pancreatic trypsinogen, while the trypsinogen concentration of the pancreatic juice was depressed below normal after stimulation. They suggested that an early action of ethionine may be one of impairing secretion of pancreatic enzymes, but most of the observed effects of ethionine on pancreatic function were attributed to protein synthesis inhibition.

The results of the present experiments clearly show that ethionine impairs the response of the pancreas to secretory stimuli. When stimulation is weak, such as that produced by the amino acid or casein diets, the enzymes are secreted so slowly when ethionine is fed that, even in the complete absence of dietary methionine, they accumulate in the pancreas. Presumably, in these short experiments, methionine requirements for synthesis of new enzyme protein to replace the small amount lost to the intestine were met through mobilization of this amino acid from other tissues. Wachstein and Meisel ('51) had reported that a preliminary depletion period on a protein-free diet was necessary to consistently produce observable pancreatic lesions by ethionine injection. With ethionine and insufficient methionine in the diet, a strong pancreatic stimulus, such as SBTI, evokes slightly more secretion, but by two hours after stimulation, the response is still far from complete. However, under the continued stimulation of SBTI, the enzymes appear to leak gradually into the digestive tract, apparently, at a rate faster than they can be renewed. Consequently, 22 hours after the initial feeding, pancreatic enzymes, and especially lipase, remain below normal. When a minimal amount of methionine is available to partially counteract the effects of the inhibitor, secretion is improved as is the ability of the acinar tissue to replace active enzymes. Whether inhibition of secretory response is related to the impaired renewal of active enzyme is not known. In the acute methionine deficiency, the latter condition was produced, but not the former, which would indicate that the enzyme response to stimulation was more susceptible to some toxic effect of ethionine than to an absence of the essential amino acid.

Sidransky and Farber ('56) had reported that pancreatic protease and amylase activity increased above normal 24 hours after ethionine and carbamylcholine injections. Since subsequent carbamylcholine injections effectively depleted the enzymes, it was felt that the increased activity represented synthesis of new enzyme rather than enzyme accumulation. The results of our study also showed an increase in pancreatic amylase activity, but not protease, in ethionine-fed rats 22 hours after stimulation. However, the increase took place only when a certain amount of methionine accompanied the ethionine. The activity measured as enzyme concentration in these experiments appears to have resulted from newly formed enzyme, because this group of animals (0.8% methionine) showed considerable pancreatic enzyme depletion two hours after stimulation. Lin and Grossman ('54) had mentioned that methionine injected simultaneously with ethionine not only prevented the depression of amylase secretion in dog pancreatic juice, but increased it above control values. The reasons for this potentiating effect on amylase synthesis by ethionine and methionine remain obscure. Hokin and Hokin ('58) had shown in *in vitro* studies with pigeon pancreas slices that amylase synthesis has a low requirement for methionine, with most of the need being met, apparently, from methionine available from the tissue. Perhaps, rat pancreas amylase also has a low methionine requirement for synthesis and responds more rapidly to less supplemental methionine than the other enzymes.

The relatively low activities of intestinal enzymes produced in the methionine-deficient rats after SBTI stimulation, indicates a definite need for methionine for normal maintenance of all the digestive enzymes. The rat pancreas however, would seem to have a considerable reserve for combating severe nutritional deprivation, since it required a rather severe deficiency before any effects became noticeable.

The demand for such high concentrations of methionine, relative to the ethionine fed, necessary to relieve or prevent some of the toxic symptoms of the analogue was unexpected. A number of investigators (Sidransky and Farber, '56; Lin

and Grossman, '54; Stekol and Weiss, '51) had reported that methionine fed or injected in quantities nearly equivalent to the ethionine prevented the toxic effects. In most of the experiments, however, it was noted that the animals were fed a diet adequate in protein, so the methionine supplements must be considered as being in addition to methionine supplied by the protein and available from tissue sources. Therefore, it would seem that ethionine may not always exert its effects by directly interfering with methionine metabolism. Evidence has been presented (Levine and Tarver, '51; Rabinovitz et al., '57) that ethionine is incorporated into protein, and the possibility has been considered that a similar incorporation into enzyme proteins might render them inactive. Swendseid et al., ('53) have shown that ethionine may also serve as a source of metabolic analogues, which when formed, then interfere with specific biological reactions. Prevention of the toxicity, resulting from a preferential or irreversible formation from ethionine of such analogues or inactive enzymes, might be expected to require excessive amounts of methionine.

SUMMARY

1. Rats were allowed to eat, for 5 days, a diet containing amino acids in the approximate amounts provided by 18% casein, and containing 0.1% of ethionine and zero, 0.2, 0.4 or 0.8% of methionine. On the 6th day they were fed the diet and a pancreatic enzyme stimulant (soybean trypsin inhibitor, SBTI) by stomach tube. After two hours or 22 hours, they were killed and the pancreas and intestinal contents analyzed for lipase, protease and amylase.

2. Under normal dietary stimulation, ethionine caused enzymes to accumulate in the pancreas regardless of the level of methionine in the diet.

3. With secretion enforced by SBTI, ethionine almost completely inhibited enzyme discharge when zero, or 0.2% of methionine was present. At 0.8% of methionine, the pancreas response to SBTI was nearly normal.

4. Twenty-two hours after stimulation, ethionine with zero, 0.2 and 0.4% of methionine greatly inhibited renewal of pancreatic lipase and to a lesser extent amylase activity. Protease activity remained relatively unaffected by methionine. With 0.8% of methionine in the diet, lipase and protease activities increased to nearly normal, whereas amylase activity in the pancreas increased to twice that of the control group.

5. An acute methionine deficiency caused a depression in enzyme activity secreted into the intestine, but apparently had no effect on pancreatic enzyme discharge.

6. The results indicated that ethionine impaired pancreatic function by inhibiting secretion and quite probably synthesis of new enzyme protein. Although methionine eventually counteracted most of the effects of ethionine it took much more than could be accounted for by assuming that the analogue acted only as a specific methionine antagonist.

LITERATURE CITED

Block, R. J., and K. W. Weiss 1956 Amino Acid Handbook. Methods and Results of Protein Analysis. Charles C Thomas, Springfield, Illinois.

De Almeida, A. L., and M. I. Grossman 1952 Experimental production of pancreatitis with ethionine. Gastroenterology, 20: 554.

Farber, E., and H. Popper 1950 Production of acute pancreatitis with ethionine and its prevention by methionine. Proc. Soc. Exp. Biol. Med., 74: 838.

Farber, E., M. V. Simpson and H. Tarver 1950 Studies on ethionine. II. The interference with lipide metabolism. J. Biol. Chem., 182: 91.

Friedberg, F., H. Tarver and D. M. Greenberg 1948 The distribution pattern of sulfur-labeled methionine in the prctein and the free amino acid fraction of tissues after intravenous administration. Ibid., 173: 355.

Goldberg, R. C., I. L. Chaikoff and A. H. Dodge 1950 Destruction of pancreatic acinar tissue by DL-ethionine. Proc. Soc. Exp. Biol. Med., 74: 869.

Hokin, L. E., and M. R. Hokin 1958 Studies of pancreatic tissue *in vitro*. Gastroenterology, 36: 368.

Kalser, M. H., and M. I. Grossman 1954 Pancreatic secretion in dogs with ethionine-induced pancreatitis. Ibid., 26: 189.

Kroboth, F. J., and G. A. Hallenbeck 1954 Some effects of ethionine in the dog, with particular reference to external pancreatic secretion. Ibid., 27: 743.

Lee, N. D., and R. H. Williams 1952 Inhibition of adaptive formation of tryptophan peroxidase in rats by ethionine. Biochem. Biophys. Acta, 9: 698.

Levine, M., and H. Tarver 1951 Studies on ethionine. III. Incorporation of ethionine into rat proteins. J. Biol. Chem., *192:* 835.

Lin, T. M., and M. I. Grossman 1954 Reversal by DL-methionine of acute effect of DL-ethionine on pancreatic enzyme output in dogs. Am. J. Physiol., *176:* 377.

Lyman, R. L., and S. Lepkovsky 1957 The effect of raw soybean meal and trypsin inhibitor diets on pancreatic enzyme secretion in the rat. J. Nutrition, *62:* 269.

Popper, H., J. Dela Huerga and D. Koch-Weser 1952 Hepatic and pancreatic changes produced in rats by ethionine and their relation to human lesions. Am. J. Path., *28:* 518.

Rabinovitz, M., M. E. Olsen and D. M. Greenberg 1957 Characteristics of the inhibition by ethionine of the incorporation of methionine into proteins of the Ehrlich ascites carcinoma *in vitro.* J. Biol. Chem., *227:* 217.

Sidransky, H., and E. Farber 1956 The effects of ethionine upon protein metabolism in the pancreas of rats. Ibid., *219:* 231.

——— 1958 Sex difference in induction of periportal fatty liver by methionine deficiency in the rat. Proc. Soc. Exp. Biol. Med., *98:* 293.

Simmonds, S., E. Keller, J. P. Chandler and V. duVigneaud 1950 The effect of ethionine on transmethylation from methionine to choline and creatine *in vivo.* J. Biol. Chem., *183:* 191.

Simpson, M. V., E. Farber and H. Tarver 1950 Studies on ethionine. I. Inhibition of protein synthesis in intact animals. Ibid., *182:* 81.

Sós, J., and T. Kemény 1956 Characteristic symptoms of isoleucine, lysine and methionine deficiency in rats and dogs. Virchow's Arch. Pathol. Anat. u. Physiol., *328:* 421.

Stein, R. J., G. Kent, T. Barka and H. Popper 1960 Effect of methionine upon ethionine intoxication of the rat. Proc. Soc. Exp. Biol. Med., *103:* 210.

Stekol, J. A., and K. W. Weiss 1951 Inhibition of growth of C3H mice by ethionine and its alleviation by methionine or choline. Ibid., *77:* 213.

Swendseid, M. E., A. L. Swanson and F. H. Bethell 1953 Ethionine as an inhibitor of enzyme systems mediating choline oxidation. J. Biol. Chem., *201:* 803.

Wachstein, M. and E. Meisel 1951 Protein depletion enhances pancreatic damage caused by ethionine. Proc. Soc. Exp. Biol. Med., *77:* 569.

——— 1954 Relation of dietary protein levels to pancreatic damage in the rat. Ibid., *85:* 314.

Wheeler, J. E., F. D. W. Lukens and P. Gyorgy 1949 Studies on the localization of tagged methionine within the pancreas. Ibid., *75:* 187.

Investigation of Precursors of Ruminal Fatty Acids of Sheep Fed Purified Diets[1,2,3]

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Recent reports have indicated that the amounts and relative proportions of short-chain fatty acids in the rumen exert numerous effects on the metabolism of the animal. Balch and Rowland ('59) showed that the amounts and proportions of fatty acids found in the rumen of lactating cows affect the milk fat levels and Ensor et al. ('59) found that diets which yielded high levels of propionic acid increased the efficiency of weight gains in fattening steers. The amounts and ratios of the volatile fatty acids in the rumen also affect the heat increment (Armstrong and Blaxter, '57; Armstrong et al., '57) and ketone body production (Johnson, '51; Clark and Malan, '56). In *in vitro* experiments, it has been shown that the metabolism of any one of the short-chain fatty acids may be modified considerably by the presence or absence of one or more of the other steam-volatile acids (Pennington and Pfander, '57; Pritchard and Tove, '60; Pennington and Appleton, '58).

The foregoing studies serve to point out the need for further elucidation of the fundamental physical and biochemical phenomena underlying the production, absorption and metabolism of the short-chain fatty acids by ruminants. In the past, the complexity and variation of normal ruminant diets have complicated the study of these basic principles. However, the recent development of roughage-free purified diets for ruminants (Matrone et al., '59) allows the addition or deletion of chemically defined components to the diet of the animal and provides a unique means for the study of some basic nutritional problems. The results reported herein represent an attempt by the authors to obtain additional information about the formation of short-chain fatty acids in the rumen of sheep fed purified diets via the *in vivo* and *in vitro* study of precursors of these acids with the view that the information would help explain the superior performance of animals fed diets containing added sodium and potassium.

EXPERIMENTAL

Two yearling wethers, raised from approximately two months of age with different purified diet regimens (Matrone et al., '59) served as the experimental subjects. The primary difference between the two diets was the presence or absence of the bicarbonates of sodium (Na) and potassium (K). Diet 1 consisted of casein, starch, glucose, vegetable oil, minerals and vitamins, and diet 1(Na plus K) contained, in addition to these ingredients, 7.3% of Na bicarbonate and 4.4% of K bicarbonate. At the initiation of this study, animal 214, receiving diet 1, weighed 48 pounds, and sheep 215, receiving diet 1(Na plus K), weighed 125 pounds. During the course of this investigation, sheep 214 was fed at a rate of 1.4 pounds per day and sheep 215 at a rate of 2.4 pounds per day.

In the *in vivo* experiments, the isotope (either $NaHC^{14}O_3$ or $C^{14}H_3COONa$) was injected directly into the rumen by needle and syringe one hour after feeding. Samples of the rumen contents were withdrawn

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[3] The data presented in this paper were taken from a thesis presented by Darrell R. Van Campen to the Faculty of the Graduate School of North Carolina State College in partial fulfillment of the requirements for the degree of Master of Science.

by stomach tube at predetermined intervals and strained through cheesecloth. Bacterial action was stopped by the addition of aqueous mercuric chloride. The samples were centrifuged and then refrigerated until analyzed for volatile fatty acid and isotope content.

The *in vitro* experiments involved the anaerobic incubation, at 39°C, of 20 ml of whole rumen fluid, taken from one or the other of the experimental animals, with the isotopic compound under study. Aliquots of the incubation mixture were removed at 0.5, 1, 2 and 4 hours after addition of the isotope to the flasks containing the rumen fluid and reaction was stopped by the addition of 0.5 ml of 20% sulfuric acid.

The amounts of short-chain fatty acids in the rumen fluid obtained in the *in vivo* studies and in the reaction mixtures of the *in vitro* experiments were determined by the chromatographic method of Keeney ('55) and the fractions obtained by this method were subsequently counted. In the plating and counting procedure, each fraction was made alkaline (pH 10 or above) and evaporated to dryness on a steam bath. The residue was resuspended in 2 ml of an aqueous suspension containing 3 mg/ml of Super-cel and 0.2 mg/ml of cellulose gum. The suspension was plated over an area of 6.15 cm^2 on stainless steel planchets which had been etched with aqua regia. After drying, the samples were counted in a windowless, gas-flow, proportional counter. Corrections for self-absorption were made by use of a standard self-absorption curve prepared by the constant activity method of Comar ('55).

RESULTS AND DISCUSSION

Bicarbonate-CO_2 studies. The bicarbonate-CO_2 studies were conducted using both *in vivo* and *in vitro* techniques. In the *in vivo* experiments, the animal receiving diet 1(Na plus K) was injected with 910 μc of $NaHC^{14}O_3$ and the sheep receiving diet 1 was given 600 μc of this material. In the *in vitro* studies, 20-ml samples of rumen fluid taken from these two sheep were individually incubated in the presence of an amount of $C^{14}O_2$ sufficient to yield 10^7 counts per minute per flask. The results

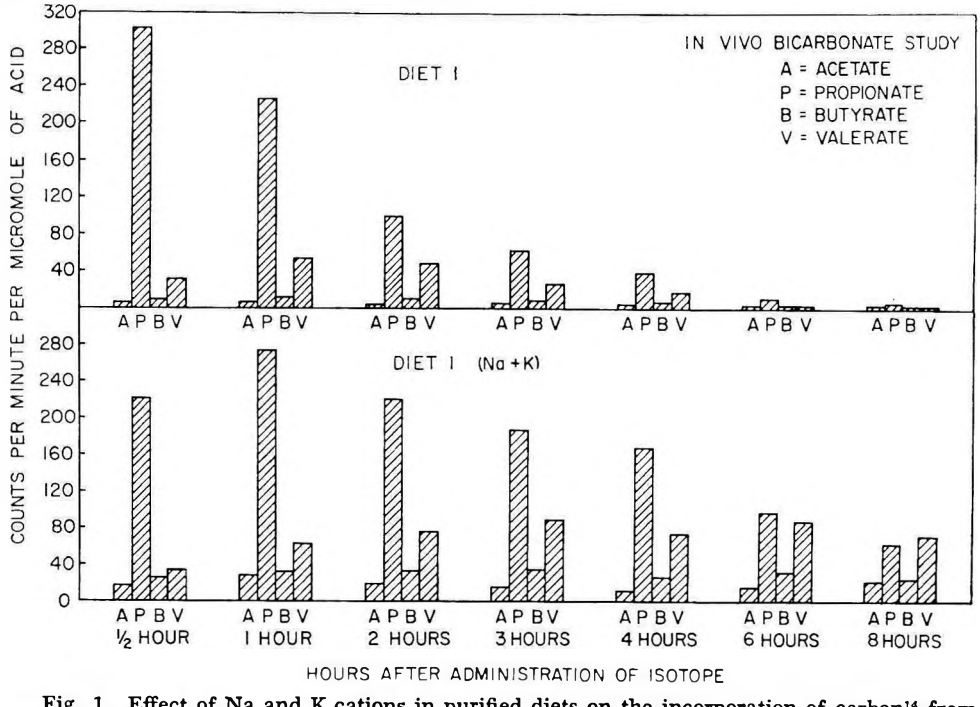

Fig. 1 Effect of Na and K cations in purified diets on the incorporation of carbon^{14} from $NaHC^{14}O_3$ into ruminal short-chain fatty acids.

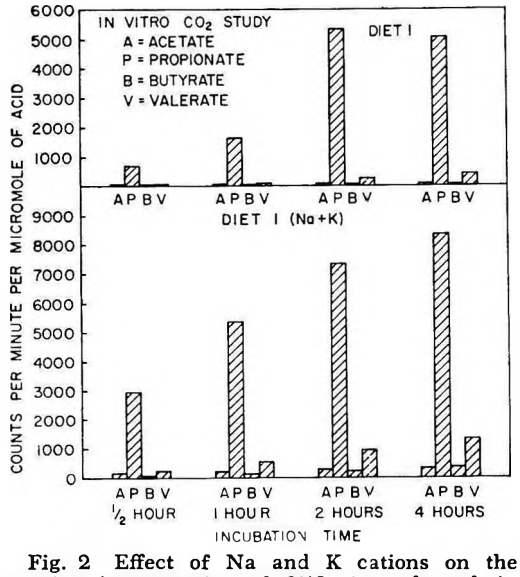

Fig. 2 Effect of Na and K cations on the *in vitro* incorporation of $C^{14}O_2$ into short-chain fatty acids by rumen microorganisms.

of the isotope analyses in these *in vivo* and *in vitro* studies are presented in figures 1 and 2, respectively.

There are some similarities between diets in the results obtained in both the *in vitro* and *in vivo* studies. The propionate contained a major portion of the radioactive labeling and the specific activity of valerate was higher than that of either acetate or butyrate irrespective of technique or diet. The large amount of labeling in propionate may be partially accounted for by the operation of several known pathways in which the end result could be the incorporation of $C^{14}O_2$ into this compound (Wood and Werkman, '40; Ochoa, '51; Flavin et al., '55). That the specific activities of acetate and butyrate are lower than that of valerate might be expected in light of the high specific activity of propionate and the fatty acid synthesis scheme as outlined by Green ('55) and James et al. ('56).

The *in vivo* studies also suggest a difference in the rate of incorporation of the labeled carbon into the fatty acids. Propionate and valerate attained peak values at one hour and three hours, respectively, after the administration of $C^{14}O_2$ in the sheep receiving diet 1(Na plus K), whereas maximum values for these two acids were observed at one-half hour and two hours, respectively, in the sheep receiving diet 1. This difference is also indicated in the *in vitro* studies in that the propionate activity in the flask containing rumen fluid from the sheep fed diet 1 appeared to reach a maximum within two hours while the corresponding activity with diet 1(Na plus K) appeared still to be on the increase at the end of the 4-hour incubation period. Percentage fixation calculations on the *in vitro* data revealed a more than twofold greater incorporation of $C^{14}O_2$ into the volatile fatty acids in the rumen fluid taken from the sheep fed diet 1(Na plus K). This difference presumably was due to the presence of the sodium and potassium bicarbonates in the diet.

Acetate experiments. In the *in vivo* acetate studies, 250 μc of acetate-2-C^{14} in aqueous solution were introduced into the rumen of each sheep, and samples of the rumen fluid were again taken at intervals of 0.5, 1, 2, 3, 4, 6 and 8 hours after administration of the isotope and analyzed for volatile fatty acid and isotope content. In the *in vitro* acetate studies, 20 ml of whole rumen fluid taken from each sheep were individually incubated in the presence of a solution of acetate-2-C^{14} containing 6.93×10^6 cpm. The results of these *in vivo* studies are given in figure 3 and

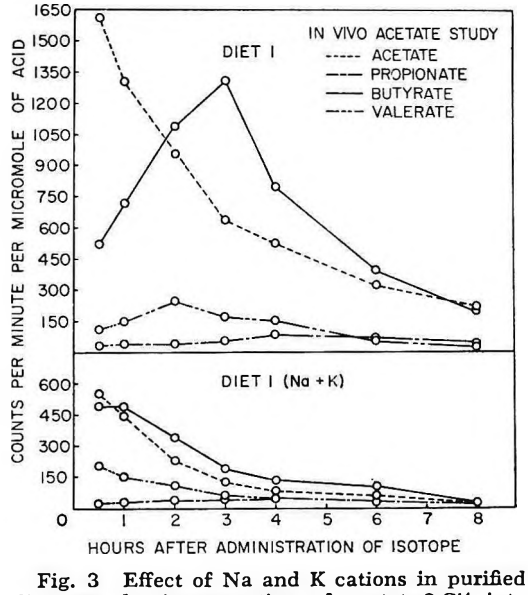

Fig. 3 Effect of Na and K cations in purified diets on the incorporation of acetate-2-C^{14} into ruminal short-chain fatty acids.

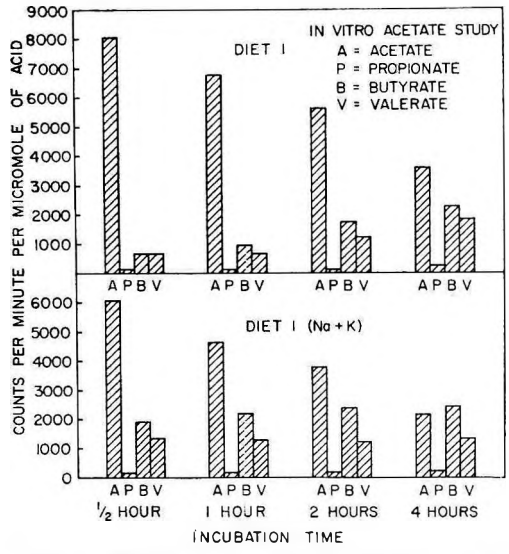

Fig. 4 Effect of Na and K cations on the *in vitro* incorporation of acetate-2-C^{14} into short-chain fatty acids by rumen microorganisms.

the *in vitro* results are presented in figure 4.

The most striking observation in these studies was the well-delineated product-precursor type curve (Zilversmit et al., '43; Hevesy, '48) which was obtained from the specific activities of acetate and butyrate in the *in vivo* studies with the sheep receiving diet 1(Na plus K). The curve obtained in the *in vivo* study involving diet 1 did not show this relationship, i.e., the specific activity versus time curve for acetate did not pass through the peak of the butyrate curve. One possible explanation for this disparity between the two sheep involves the accumulation of intermediates. Radin ('48) has shown that if intermediates are present, the specific activity-time curves do not display a typical product-precursor relationship. In the proposed pathways for butyrate synthesis from acetate (Green, '55; Wakil, '58) several known intermediates occur. Thus the possibility arises that the synthesis of butyrate from acetate takes place in the rumen of the sheep receiving diet 1(Na plus K) without a significant accumulation of intermediates, whereas one or more intermediates accumulated during butyrate synthesis in the rumen of the sheep fed diet 1 because of a rate-limiting step. That the rate-limiting process might be a univalent cation requiring reaction is consistent with the results reported by Hiatt and Evans ('60) on the cofactor requirements of aceto-CoA-kinase. This would then preclude the possibility of a typical product-precursor relationship. Other factors including differential production and absorption of the fatty acids and the possible lack of steady state conditions may present other alternatives. It can also be seen that the specific activity of butyrate and valerate reach maximum values sooner in the rumen fluid of sheep 215 (diet 1, Na plus K) than in sheep 214 (diet 1) irrespective of whether the *in vivo* or the *in vitro* technique was used.

Production of short-chain fatty acids. The results of the *in vivo* and *in vitro* analyses are presented in figures 5 and 6, respectively. The interesting comparison between the *in vivo* fatty acid production (fig. 5) from the biologically successful diet 1(Na plus K) and the biologically unsuccessful diet 1 reveal two major differences. The first of these is the comparison of the relative levels of propionate and acetate in the two animals, i.e., the level of propionate in the rumen fluid of the sheep receiving diet 1(Na plus K) approached and in some cases exceeded the level of acetate, whereas in the other animal the level of propionate was consistently less than one-half that of acetate. The other major difference was that the levels of all of the acids in the rumen fluid of the sheep receiving diet 1 decreased more rapidly with time after feeding than was the case with the sheep fed diet 1(Na plus K). In the *in vitro* studies (fig. 6), butyrate concentrations increased at a more rapid rate in the incubations using rumen fluid from the sheep fed diet 1(Na plus K) than in incubations using rumen fluid from the other animal. This observation provides some indirect evidence in favor of the foregoing interpretation of the product-precursor relationships observed. Another interesting observation was the presence of large amounts of the acids with chain lengths of 6 carbon atoms or more in the *in vitro* fermentations involving diet 1.

In conclusion, it appears that a major role of CO_2 in the formation of ruminal volatile fatty acids is as a precursor of

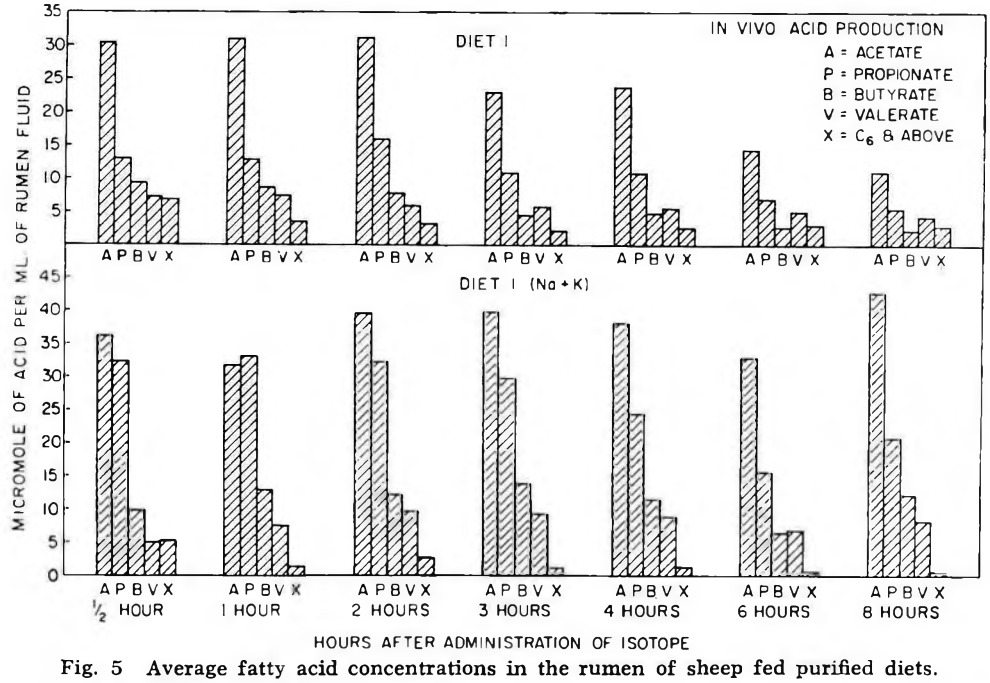

Fig. 5 Average fatty acid concentrations in the rumen of sheep fed purified diets.

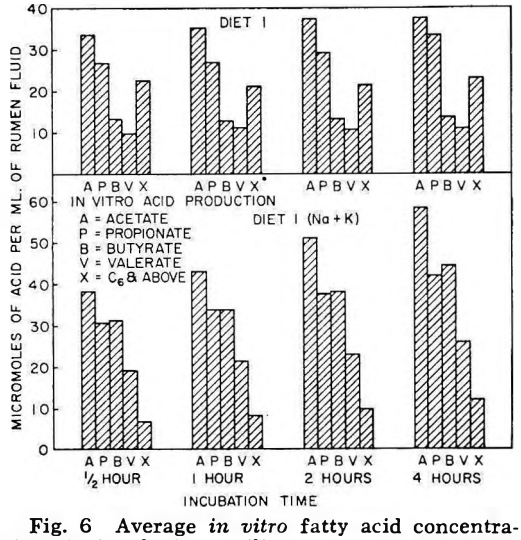

Fig. 6 Average *in vitro* fatty acid concentrations in incubations utilizing rumen microorganisms from sheep fed purified diets.

propionate and subsequently of valerate whereas acetate is a major precursor of both butyrate and valerate. The incorporation of either of these materials into their volatile fatty acid products was enhanced by the presence of sodium and potassium bicarbonates in the diet. It seems that two separate metabolic pathways were involved, one for propionate and the other for acetate and butyrate. The data on the fate of radioactivity from either CO_2 or acetate in respect to valerate, suggesting that one pathway in the formation of valerate involved the condensation of a two-carbon unit with a three-carbon unit is consistent with the observations of Bornstein and Barker ('48) and Ladd ('59).

SUMMARY

The *in vivo* and *in vitro* incorporation of C^{14}-labeled bicarbonate and acetate-2-C^{14} into the ruminal volatile fatty acids of sheep receiving purified diets was studied. In the bicarbonate-CO_2 studies, most of the incorporated label was found in propionate and the specific activity of valerate exceeded that of either acetate or butyrate in all cases. A difference in incorporation rates between the sheep receiving diet 1(Na plus K) and the sheep fed diet 1 was suggested and the addition of sodium and potassium bicarbonates to the diet enhanced $C^{14}O_2$ incorporation into the fatty acids in the *in vitro* studies. A product-precursor type relationship between acetate and butyrate was encountered in the

in vivo experiments and this relationship received additional support from the fatty acid analyses. Here again, the presence of the supplementary sodium and potassium bicarbonates enhanced the incorporation of C^{14}. Volatile fatty acid analyses revealed relatively higher levels of propionate in the rumen fluid of the sheep receiving diet 1(Na plus K) than observed in rumen fluid from the sheep fed diet 1.

LITERATURE CITED

Armstrong, D. G., and K. L. Blaxter 1957 The heat increment of steam-volatile fatty acids in fasting sheep. Brit. J. Nutrition, *11:* 247.

Armstrong, D. G., K. L. Blaxter and N. McC. Graham 1957 Utilization of steam-volatile fatty acids. Ibid., *11:* 392.

Balch, C. C., and S. J. Rowland 1959 Studies of the secretion of milk of low fat content by cows on diets low in hay and high in concentrates. VII. The effect of administration of volatile fatty acids to cows giving normal milk and milk of low fat content. J. Dairy Res., *26:* 162.

Bornstein, B. T., and H. A. Barker 1948 The energy metabolism of *Clostridium kluyveri* and the synthesis of fatty acids. J. Biol. Chem., *172:* 659.

Clark, R., and J. R. Malan 1956 Alteration in blood sugar and ketone levels caused by dosing acetate, propionate and butyrate into the rumen of sheep. Ond. J. Vet. Res., *27:* 101.

Comar, C. L. 1955 Radioisotopes in Biology and Agriculture. McGraw-Hill Book Co., Inc., New York.

Ensor, W. L., J. C. Shaw and H. F. Tellechea 1959 Special diets for the production of low fat milk and more efficient gains in body weight. J. Dairy Sci., *42:* 189.

Flavin, M., P. J. Ortiz and S. Ochoa 1955 Metabolism of propionic acid in animal tissues. Nature, *176:* 823.

Green, D. E. 1955 Oxidation and synthesis of fatty acids in soluble enzyme systems of animal tissues. Clinical Chem., *1:* 53.

Hevesy, G. 1948 Radioactive Indicators. Interscience Publishers, Inc., New York.

Hiatt, A. J., and H. A. Evans 1960 Influence of cations on the activity of aceto-CoA-kinase from spinach leaves. Plant Physiol., in press.

James, A. F., G. Peeters and M. Lauryssens 1956 The metabolism of propionic acid. Biochem. J., *64:* 726.

Johnson, R. B. 1951 The relative rates of absorption of the volatile fatty acids from the rumen and their relationship to ketosis. Cornell Vet., *41:* 115.

Keeney, M. 1955 Direct chromatographic determination of C_1 to C_6 fatty acids in rumen fluid. Univ. Maryland, Misc. Pub. 238, p. 23.

Ladd, J. N. 1959 The fermentation of lactic acid by a gram-negative coccus. Biochem. J., *71:* 16.

Matrone, G., H. A. Ramsey and G. H. Wise 1959 Effect of volatile fatty acids, sodium and potassium bicarbonates in purified diets for ruminants. Proc. Soc. Exp. Biol. Med., *100:* 8.

Ochoa, S. 1951 Biological mechanisms of carboxylation and decarboxylation. Physiol. Rev., *31:* 56.

Pennington, R. J., and J. M. Appleton 1958 Further studies on the inhibition of acetate metabolism by propionate. Biochem. J., *69:* 119.

Pennington, R. J., and W. H. Pfander 1957 The metabolism of short-chain fatty acids in the sheep. 5. Some interrelationships in the metabolism of fatty acids and glucose by sheep-rumen epithelial tissue. Ibid., *65:* 109.

Pritchard, G. I., and S. B. Tove 1960 Interrelationships between the metabolism of short-chain fatty acids by ruminant liver slices. Biochim. Biophys. Acta, in press.

Radin, N. S. 1948 Isotope Techniques in Biochemistry. U. S. Atomic Energy Commission, Isotopes Division, Oak Ridge.

Wakil, S. J. 1958 A malonic acid derivative as an intermediate in fatty acid synthesis. J. Am. Chem. Soc., *80:* 6445.

Wood, H. G., and C. H. Werkman 1940 The fixation of CO_2 by cell suspensions of *Propionibacterium pentosaceum*. Biochem. J., *34:* 7.

Zilversmit, D. B., C. Entenman and M. C. Fisher 1943 On the calculation of "turnover time" and "turnover rate" from experiments involving the use of labeling agent. J. Gen. Physiol., *26:* 325.

The Effects of L-Thyroxine and Cold-Exposure on the Amount of Food Consumed and Absorbed by Male Albino Rats

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Rats exposed to a cold environment or rendered hyperthyroidic by daily injections of thyroid hormone increase their food intake. It would be interesting to know if the energy available for utilization by the animals, under these conditions, is proportional to the amount of food eaten. Determination of the digestion coefficients of the food consumed during cold exposure or while hyperthyroidic would provide the information. The digestion coefficient of a diet represents the number of grams of food digested and absorbed by an animal for each 100 gm of food eaten, and is important in determining the nutritive value of a diet. When the diet is kept constant, changes in the digestion coefficient indicate changes in the ability of the animal to digest and absorb the food. This paper reports the results of such a study.

METHODS

Eight male albino rats were used. They were kept in individual metal cages and fed ground laboratory chow[1] (containing about 1% of chromic oxide) and water ad libitum.

During the first part of the experiment the animals were kept in a temperature of 28°C. After three weeks at this temperature, 4 of the rats were given daily subcutaneous injections of 20 μg of L-thyroxine for two weeks. The two-week postinjection period was followed by the transfer of all the animals to a cold room maintained at 3 to 4°C. After 8 weeks in the cold the 5 surviving animals were given daily subcutaneous injections of 20 μg of L-thyroxine for two weeks. The experiments were terminated two weeks after the last injection.

At about the same hour each morning, body weights and food consumption were recorded to the nearest gram. Digestion coefficients (DC) were determined weekly by the method described in detail by Schürch and co-workers ('50).

RESULTS

Experiments at 28°C

The DC of the rats are shown in table 1. The 7-week average DC of the control group was 78.4%. One week after the beginning of daily injections of thyroxine the DC of the test group was significantly smaller.

Table 2 shows the effects of daily doses of 20 μg of L-thyroxine on food intake and body weight. During the first week there was a small increase in the amount of food eaten but none in the food absorbed (due to reduction in DC). The body weights of the rats receiving thyroxine remained stationary for about one week. As this retardation of growth appeared within 24 hours it may not have been due to increased heat production. During the second week there were marked increases in the amounts of food eaten and absorbed. Body weights increased at approximately the same rate in both groups during this period. The heat production of the test group, determined by oxygen consumption measurements, was found to be 42% greater than that of the control group. Therefore the food intake must have been sufficient to maintain this high rate of heat production and to provide enough calories for weight increase. During the first week of the postinjection period both food intake and food absorption were reduced, but the quantities remained greater than those of the

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[1] Purina Laboratory Chow.

TABLE 1

Digestion coefficients of a standard diet fed to rats kept at 28°C and given daily subcutaneous injections of 20 μg of L-thyroxine for two weeks[1]

Group	No. of rats	Control period			Injection period		Postinjection period	
		1st week	2nd week	3rd week	1st week	2nd week	1st week	2nd week
Control	4	79.6 ± 0.83[2]	78.5 ± 1.12	77.1 ± 1.09	77.9 ± 0.45	78.9 ± 2.20	78.3 ± 0.69	78.4 ± 0.70
					$P < 0.01$			
Test	4	78.3 ± 0.97	75.4 ± 1.47	76.5 ± 1.62	71.4 ± 1.24	74.1 ± 0.98	77.1 ± 0.79	79.1 ± 0.42
				$P < 0.01$		$P < 0.01$		

[1] Digestion coefficients that are significantly different are connected by braces.

[2] Standard error indicated by ±.

control group. The greater growth rate of the test group during this week suggested a possible reduction in their heat production. This was confirmed by finding that the oxygen consumption of the test group was now only 1% greater than that of the control group (not significant). During the second postinjection week although the amount of food eaten and absorbed decreased further, it still remained somewhat higher than that of the controls.

Experiments at 4°C

During exposure to cold three rats died. This is approximately the death rate of our rats kept at 4°C and fed laboratory chow.[2] We believe death was due to cold injury rather than to inability of the animals to adapt metabolically to the lower temperature. All the rats developed edema of the hind limbs during the first week of exposure but this disappeared during the second week. During the third and 4th weeks their tails became edematous and wet. The animals invariably bit off varying amounts of the tips of their tails and bleeding occurred in some cases. Rats in which bleeding occurred were usually dead by the next day. This condition was not caused by the chromic oxide in the food since we have found it to occur in animals fed this chow alone. Apparently it was the result of the combined effects of cold and the diet, since rats fed this chow at a slightly higher temperature (6°C) and rats fed another type of chow but kept at 3 to 4°C did not suffer from this condition.

The average daily food intake, weekly DC and weekly changes in body weight of the 5 surviving rats are shown in table 3. There was a small but significant fall of DC when the rats were placed in the cold. The average DC for the first 8 weeks was 75.8%. The injection of thyroxine was followed by a further decrease in DC to about 73.8% but this did not occur until two weeks after the beginning of injections. Although the same amounts of food were absorbed during the first and second weeks of thyroxine administration, the growth rate was retarded only during the first week.

[2] Purina Laboratory Chow.

TABLE 2

Average daily food intake and weekly changes in body weight of rats kept at 28°C and given daily subcutaneous injections of 20 μg of L-thyroxine for two weeks

Group[1]	Injection period				Postinjection period			
	First week		Second week		First week		Second week	
	Control	Test	Control	Test	Control	Test	Control	Test
Food intake, gm/day	14.58[2]	15.80	13.45	19.02	14.38	16.83	13.08	14.88
			$P < 0.001$		$P < 0.05$			
Food absorbed gm/day	11.36	11.28	10.61	14.10	11.26	13.05	10.25	11.77
			$P < 0.001$		$P < 0.05$			
Body weight	221.75	212.25	228.25	219.75	234.75	231.75	240.5	240.25
Weight change gm/week	+8.5	+0.5	+6.5	+7.5	+6.5	+12.0	+5.75	+8.25
	$P < 0.001$				$P < 0.05$			

[1] Four animals per group.

[2] The standard errors have been omitted to simplify the table. Values significantly different are connected by braces.

[3] Food absorbed indicates food eaten × DC/100. (DC obtained from table 1.)

The regression of change in body weight of the rats, on food intake is curvilinear (fig. 1). This figure is of interest because during two weeks no change in body weight occurred. Thus the amount of food eaten during this period (28.4 gm/day) supplied the energy required for weight maintenance at 4°C. This type of data cannot be obtained from rats fed ad libitum in a warm environment. The expected relationship between body weight change and food intake is a linear one. In rats, heat production increases gradually during the first one or two weeks of exposure to cold (Cottle and Carlson, '54). If we disregard the first two weeks of cold exposure in the present experiments a linear regression of body weight change on food consumption is obtained ($r = 0.954$; significant at the 1% level). The reciprocal of the slope of this line is 1.94 gm of food eaten per gm of body weight increase. Correcting for unabsorbed food (DC = 75.8%) and taking 3.94 Cal./gm as the caloric value of the diet, the caloric equivalence of the tissues formed is 5.79 Cal./gm. Similar calculations give rise to a maintenance requirement at 4°C of 84.82 Cal./day.

DISCUSSION

The method used to determine the digestion coefficients involves the incorporation in the food of an indigestible material (chromic oxide) and estimation of the degree to which it has been concentrated in the feces. The formula used is: $DC = 100(1-B/A)$; where A represents the parts of nutrient per unit of chromic oxide in the diet and B the parts of nutrient per unit of chromic oxide in the feces. Digestion coefficients should not be taken literally. The main sources of error in the practical application of this formula are: (1) the use of dry weight of feces to indicate the amount of nutrient in the feces; and (2) the eating of feces by the rats. In many instances the fecal material is preponderantly endogenous and bacterial (e.g., in starvation). Since the amount of bacteria in the feces must contribute significantly to the dry weight, this factor would tend to reduce the DC. Recirculation of the index material resulting from coprophagy would tend to increase the DC. Thus, in addition to assuming that the index material is thoroughly mixed in both food and feces we must assume that the concentration of bacteria in the feces and the degree of coprophagy remain constant under the different experimental conditions.

Our results suggest that thyroxine has separate effects on food absorption and on food consumption. At 28°C a reduction in

TABLE 3

Average daily food intake, digestion coefficients and body weights of 5 rats kept at 4°C and given daily subcutaneous injections of 20 μg of L-thyroxine for two weeks[1]

	28°C	Exposure to 4°C (weeks)						Injection period			
		1	2	3	4	5	8	9	10	11	12
Food intake, gm/day	13.60	22.28	26.10	27.46	28.24	28.68	30.60	31.27	31.83	32.20	30.73
Digestion coefficients	78.42	75.84	76.78	75.26	76.24	75.54	75.38	75.28	73.78	76.04	76.28
		$P < 0.01$						$P < 0.01$	$P < 0.01$		
Food absorbed, gm/day[2]	10.66	16.90	20.04	20.67	21.53	21.66	23.07	23.54	23.48	24.48	23.44
Body weight, gm	239.6	229.8	225.4	224.4	222.2	222.2	242.2	242.6	248.8	256.8	262.4
Weight change, gm/week	+9.4	−9.8	−4.4	−3.0	−0.2	0	+7.4	+0.4	+6.2	+8.0	+5.6

[1] Standard errors have been omitted to simplify the table. Digestion coefficients that are significantly different are connected by braces.
[2] Food absorbed indicates food eaten × DC/100.

Fig. 1 Regression of change in weight of rats, on their food intake during exposure to a room temperature of 4°C. Each point represents the daily average from 5 rats during a one-week period. The numbers near the points indicate the number of weeks of exposure to cold.

DC is followed by an increase in food consumption. When the injections of thyroxine are stopped the return to preinjection levels follows the same sequence. When thyroxine is injected into cold-adapted rats (8 weeks at 4°C) the reduction in DC is smaller and occurs after two weeks; there is no increase in food consumption.

The reduced responses to L-thyroxine in rats exposed to cold needs explanation. This is possible if we assume that the injected thyroxine must first be converted to an active form and that it is the concentration in the plasma of this active form that determines the degree of the response. The estimated daily requirement for L-thyroxine is about 3.5 μg for rats kept at 25°C and 7.5 μg for those kept at 5°C (Woods and Carlson, '56). The increased requirement for thyroid hormone in rats exposed to cold is met by an increase in rate of production (Cottle and Carlson, '56) but there is no increase in plasma concentration of the hormone (Rand et al., '52). Thus injection of equal doses of L-thyroxine into cold-exposed and warm-exposed rats does not result in higher

plasma concentrations of L-thyroxine in those exposed to cold.

On the other hand, a given dose of thyroxine-1^{131} has been found to leave the plasma and to be excreted more rapidly in cold exposed animals.[3] The increased rate of disappearance of L-thyroxine from the plasma would reduce the rate of its conversion to the active form. Another factor would come into play if the dose of injected L-thyroxine is sufficient to inhibit endogenous secretion. The higher rate of utilization of the active form in rats exposed to cold would lead to a lower final concentration.

The thyroid gland has been implicated in various aspects of the digestive functions of animals (Watman and Nasset, '51; Thompson and Vars, '54; Russel and Nasset, '53). However, the reduction in DC observed in our experiments could have been the result of an increase in gastrointestinal peristalsis.

The reduction in growth rate of the rats after L-thyroxine injections appears early (within 24 hours). It is of short duration and occurs to the same degree in both warm- and cold-exposed rats. These facts suggest that the reduction in growth rate may be a separate effect of L-thyroxine. It could result from a transient disturbance of water balance (increased rate of excretion or reduced intake).

The caloric requirement for weight maintenance theoretically varies directly with rate of heat loss. Under similar environmental conditions heat loss from an animal varies directly with its surface area and inversely with its insulation. Chiu and Hsieh ('60) have observed that, at 25°C, the absolute heat production of rats with body weights varying between 200 gm and 300 gm are approximately the same. It would seem, therefore, that in rats in this weight range the increase in surface area that is associated with increase in body weight is in some way accompanied by an increase in insulation. In the present experiments the surface area of the rats increased by about 5.8% from the third to the 8th week of cold exposure. Our calculation of the caloric requirement for weight increase is based on the assumption that the small increase in surface area of the animals has not affected the caloric requirement for maintenance of body weight.

Kaunitz et al., ('57), by restricting food consumption to the amount necessary for weight maintenance and then noting the increase in weight and the food consumption during the first three days of ad libitum feeding, have estimated the food requirements for growth in rats to be about 1 gm of food eaten for 1 gm of body substance formed. A possible source of error in the results of Kaunitz et al. ('57) is the use of gain in weight during the first three days of re-alimentation as an indication of increase in body substance. A considerable amount of the increase in body weight must have been due to an increase in intestinal contents following re-alimentation. We have placed 4 rats on a restricted diet for 4 weeks and have found that after three days of ad libitum feeding the average increase in body weight was 31 gm, giving an average rate of increase of about 10 gm/day. However, 19.5 gm of the total increase occurred during the first day and the rate of increase of body weight for the subsequent days was only about 5 gm/day. We therefore believe that our value of 1.9 gm/gm of body weight increase is a closer approximation to the food requirements for growth in rats.

SUMMARY

1. The effects of environmental temperature and daily subcutaneous injection of 20 μg of L-thyroxine on the digestion coefficient (DC) of a standard diet fed to rats was determined by the use of chromic oxide as an index material.

2. At 28°C the DC was about 78.4%. The injection of L-thyroxine resulted in a reduction of the DC to about 71.4%.

3. At 4°C the DC was about 75.8%. The injection of L-thyroxine resulted in a slight, but significant, fall of DC to about 73.8%.

4. Food consumption of rats kept at 28°C increased after daily injections of L-thyroxine for two weeks, but this did not occur when the experiment was repeated at 4°C.

5. It is suggested that L-thyroxine may have had separate effects on food consumption and on the ability of the animals to

[3] Unpublished data quoted by Woods and Carlson ('56).

absorb food. The reduction in DC could have been the result of increased peristaltic activity.

6. For rats weighing about 220 gm the energy requirement for maintenance of body weight at 4°C was about 34.8 Cal./day. The caloric equivalence of the tissues formed was about 5.8 Cal./gm.

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LITERATURE CITED

Chiu, C. C., and A. C. L. Hsieh 1950 A comparative study of four means of expressing the metabolic rate of rats. J. Physiol., *150:* 694.

Cottle, M., and L. D. Carlson 1956 Turnover of thyroid hormone in cold-exposed rats determinated by radioactive iodine studies. Endocrinology, *59:* 1.

Cottle, W., and L. D. Carlson 1954 Adaptive changes in rats exposed to cold. Caloric exchange. Am. J. Physiol., *178:* 305.

Kaunitz, H., C. A. Slanetz and R. E. Johnson 1957 Utilization of food for weight maintenance and growth. J. Nutrition, *62:* 551.

Rand, C. C., D. S. Riggs and N. B. Talbot 1952 The influence of environmental temperature on the metabolism of thyroid hormone in the rat. Endocrinology, *51:* 562.

Russel, R. A., and E. S. Nasset 1953 Influence of the thyroid gland on jejunal secretion in the dog. Am. J. Physiol., *172:* 100.

Schürch, A. F., L. E. Lloyd and E. W. Crampton 1950 The use of chromic oxide as an index for determining the digestibility of a diet. J. Nutrition, *41:* 629.

Thompson, J. C., and H. M. Vars 1954 Influence of thyroid activity on the hepatic excretion of cholic acid and cholesterol. J. Appl. Physiol., *179:* 405.

Watman, R. N., and E. S. Naset 1951 Evidence for a non-thyroid factor which affects gastric function. Am. J. Physiol., *166:* 131.

Woods, R., and L. D. Carlson 1956 Thyroxine secretion in rats exposed to cold. Endocrinology, *59:* 323.

Experimentally Induced Muscular Dystrophy: Blood Creatine Levels and Histopathological Changes in Dystrophic Rabbits[1]

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Muscular dystrophy is a disease primarily of the young. It affects lambs, calves and even humans. Although this disease is not localized, in farm animals it occurs to a greater extent in certain areas. The Northwest has an exceptionally high incidence of muscular dystrophy in both lambs and calves. A survey (Galgan et al., '56) indicated that over 10% of all nutritional diseases of cattle in Washington were attributed to muscular dystrophy.

Unfortunately, it is often too late for therapeutic treatment when visible symptoms of muscular dystrophy are first observed. Therefore, a need for a test that would indicate its early presence is obvious. A test, in which muscular dystrophy was produced under different dietary regimes, was conducted to determine whether blood creatine is an index of dystrophic changes. Most previous work on creatine has centered around urinary creatine. Since urine starts as a kidney filtrate of the blood it would seem logical that blood creatine concentrations would be as good an indicator as urinary levels in denoting dystrophic changes because of the role of blood in transport. Melville and Hummel ('51) demonstrated a significant increase in blood creatine with the development of muscular dystrophy. Histological changes in affected muscles were observed also to substantiate or disprove the validity of creatine in indicating the presence of muscular dystrophy.

A deficiency of vitamin E in rations of lambs (Willman et al., '46), calves (Blaxter et al., '52a) and rabbits (Morgulis and Spencer, '36a) has been shown to cause muscular dystrophy. Potassium (Hove and Herndon, '55; Flipse et al., '48) and choline (Hove and Copeland, '54; Johnson et al., '51) deficiencies also have been shown to cause muscular dystrophy in experimental animals. Muscular dystrophy as a result of avitaminosis E, includes cardiac and skeletal muscular lesions in calves (Blaxter and Wood, '52b), rabbits (Madsen et al., '33) and lambs (Willman et al., '46). Deficiencies of either choline (Hove and Copeland, '54; Johnson et al., '51) or potassium (Hove and Herndon, '55) cause similar, although not identical, lesions.

The degree of creatinuria in various animals indicates the degree of muscular dystrophy (Morgulis and Spencer, '36a; Butturini, '49; Whiting et al., '49; Melville and Hummel, '51; Bacigalupo et al., '52; Blaxter et al., '53; Milman and Milhorat, '53; Hove et al., '55). Blaxter and Wood ('52) showed a decreased excretion of creatinine and an increased excretion of creatine in the urine, together with the loss of myosin, potassium and creatine from the muscle cell and an infiltration of sodium and water.

EXPERIMENTAL

Sixteen weanling male California rabbits were grouped according to weight, divided into 4 lots of 4 rabbits each, and housed individually in metal cages with raised wire floors. Previous pilot trials with rabbits had established the "normal" blood creatine level (1.53 mg/100 ml,

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TABLE 1

Percentage composition of experimental diets

Ingredients	Diet			
	Lot 1 Vitamin E-deficient	Lot 2 Potassium-deficient	Lot 3 Triethyl choline added[1]	Lot 4 Choline-deficient
Powdered cellulose[2]	20	20	20	20
Casein[3]	15	15	15	15
Dextrose[4]	49	49	49	49
Cod liver oil	3	—	—	—
Yeast, Torula	10	—	—	—
Salt mix[5]	3	3	3	3
Corn oil	—	3	3	3
Vitamin mix[6]	—	10	10	10

[1] Prepared by the method of Channon and Smith ('36).
[2] Solka Floc, Brown Company, Berlin, New Hampshire.
[3] Supplied by Mr. William Lang, International Casein Corporation, New York.
[4] Cerelose, Corn Products Refining Company, New York.
[5] Hubbel et al. ('37).
[6] A vitamin mix comparable to the B-vitamins supplied by 10% of yeast plus vitamin A and D comparable to that in 3% of cod liver oil was added to rations 3 and 4. This mixture consisted (in milligrams) of thiamin·HCl, 11; riboflavin, 171.6; niacin, 1509; pyridoxine·HCl, 79; Ca pantothenate, 155; p-aminobenzoic acid, 30; biotin, 1.3; inositol, 12.3; choline chloride (or triethyl choline), 7.6; menadione, 100; and vitamin B_{12}, trace; vitamin A, 1,634,000 IU; vitamin D, 163,400 IU; vitamin E, 15 mg/pound.

with a range from 1.29 to 1.74 mg/100 ml, and therefore, a control group was not used in this experiment. Rabbits were fed the rations presented in table 1. Distilled water was available at all times. All rabbits were supplied with a stock diet for one week before being given the experimental diets. The rabbits were weighed at the beginning of the experiment and thereafter at two-week intervals. Blood samples were taken by cardiac puncture and analyzed for whole blood creatine according to the method of Fister.[3]

The experiment was terminated on the 106th day. At death or upon sacrificing a rabbit, histopathological examinations were made of the skeletal muscles, heart and sciatic nerve.

RESULTS AND DISCUSSION

All rabbits gained weight during the first 14 days. This trend continued in lots 2 and 4 (potassium and choline deficiencies, respectively) for approximately 56 days, while in lots 1 and 3 (those vitamin E-deficient and receiving added triethyl choline, respectively) there was a decrease in body weight. In all cases there was a loss of appetite and weight before the animals exhibited paralysis or immobility. A similar syndrome has been reported by Morgulis and Spencer ('36).

The first symptoms of muscular dystrophy in lot 1 were observed 25 days after the experiment began. All of the rabbits in this lot died between 30 and 35 days. Upon autopsy, the nerves in rabbits in lot 1 were found to be normal. There was calcification, atrophy and proliferation of muscle nuclei of the cardiac musculature, accompanied by severe Zenker's degeneration of the skeletal muscle fibers. Many of the fibers were completely atrophic with only nuclei remaining. There was a loss of myofibrils and striations in nearly all fibers. A rapid rise in blood creatine occurred and was followed by sudden death (table 2).

The symptoms exhibited by rabbits in lot 2 (potassium-deficient) were noticed about 65 days after the beginning of the experiment. This was approximately twice as long after as reported by Hove and Herndon ('55) who stated that potassium deficient rabbits usually developed a severe and rapidly progressing muscular dystrophy with death occurring after 4 to 6 weeks. The potassium-deficient animals did not exhibit immobility or a complete loss of balance, but if placed on their sides

[3] Fister, Harold J. 1950 Manual of standardized procedures for spectrophotometric chemistry. Standard Scientific Supply Corp.

TABLE 2

Mean whole blood creatine in rabbits receiving different treatments

Lots	Creatine mg/100 ml					
	Day 1	Day 14	Day 28	Day 42	Day 56	Day 70
1	1.59	2.53[1]	3.11[1]			
2	1.32	1.53	1.71	1.96	2.50	2.94
3	1.28	2.01[2]	2.08	2.48[2]	2.91	3.06[2]
4	1.10	1.03	1.48	1.74	2.04	2.56

[1] $P < 0.01$, significantly higher creatine values than samples taken on day 1.
[2] $P < 0.05$, significantly higher creatine values than samples taken on day 1.

they were unable to recover their balance. The blood creatine of one rabbit rose markedly after only 14 days on the diet.

Autopsy of potassium-deficient rabbits revealed normal nerves in three of the 4 tested; the one remaining exhibited moderate degeneration of the sciatic nerve. There were scattered areas of calcification and fragmentation hyalinization and reduplication of nuclei of the skeletal muscles. Several areas of calcification, atrophy and fibrosis of the cardiac muscles were noted in one rabbit. This moderate degeneration could be the reason the animals did not exhibit immobility or complete loss of balance. A considerable overlapping of symptoms of vitamin E and potassium deficiencies was noted. This is to be expected since the end result in each case is muscular dystrophy. Triethyl choline cannot replace choline in biological processes in which choline acts as a methyl donor, but is approximately equal to choline in the prevention of fatty livers (Keston and Wortis, '46). The administration of the choline analogue was oral in this experiment and was fed at a low level; hence it was not toxic. All of the rabbits in the triethyl choline-supplemented group died within 75 days, whereas the last one fed the choline-deficient diet did not die until the 106th day. Autopsy of the rabbits in this lot showed that the skeletal muscles were severely calcified in some animals but not in others. Atrophy and a compensatory hypertrophy and loss of cross striations in many fibers, a reduplication of some nuclei, and small areas of hyalinization were also observed. These lesions were similar but not identical to those seen in calves with spontaneous muscular dystrophy. In one rabbit the sciatic nerve fibers were vacuolized and the axons had disappeared. In all of the rabbits there were foci of necrosis, and in some cases the cardiac muscles were infiltrated with neutrophils. Also a few fibers appeared hyalinized and fragmented, with proliferation of nuclei. There was no fatty infiltration of the livers.

The choline-deficient rabbits did not become completely disabled. These data agree with the findings of Hove and Herndon ('55). The blood creatine of rabbits on this treatment increased slowly but progressively (table 2).

Autopsy of the rabbits from this treatment revealed, in general, the same picture as that of triethyl choline-treated lot except that the cardiac muscles and nerves were not affected. The liver showed scars and areas of fibrosis and necrosis with an increase in interlobular connective tissue.

Table 2 shows the mean blood creatine values for each lot. In all lots the blood creatine content increased with time. The correlation of blood creatine levels with time indicated the presence of muscular degeneration. Correlation coefficients of these variables were 0.90 for lot 1, 0.74 for lot 2, 0.89 for lot 3 and 0.75 for lot 4. All of these coefficients are highly significant ($P < 0.01$). These data indicate that blood creatine levels are reliable in determining the presence of muscular dystrophy. Melville and Hummel ('51) reported that the greatest concentration of blood creatine occurs during the incipent stage when there is no histological evidence of altered structure. No animals in this experiment were sacrificed during the course of the experiment in order to note the progress of histological changes. They were autopsied either at death or the termination of the experiment. Therefore, it would be quite assumptive to conclude that these data denote severity as related to degree of degeneration.

SUMMARY AND CONCLUSIONS

Sixteen male California rabbits were divided into 4 lots of 4 each and fed semi-purified rations known to produce muscular dystrophy. Lots 1, 2 and 4 were deficient in vitamin E, potassium and choline, respectively. Triethyl choline was added to the diet of lot 3. Blood samples were obtained by cardiac puncture and whole blood creatine levels were determined. Correlation coefficients between blood creatine and time were 0.90, 0.74, 0.89 and 0.75 for lots 1 through 4, respectively. These data indicate that blood levels of creatine may be used to indicate the presence of muscular dystrophy. Considering the ease of sample collection and analysis and its high degree of accuracy, it is a very applicable method.

The most severe muscular degeneration occurred in lot 1. In lots 2 and 4 the skeletal muscles degenerated moderately and in lot 4 a slight cardiac degeneration was observed.

LITERATURE CITED

Bacigalupo, F. A., R. Culik, R. W. Luecke, F. Thorpe, Jr. and R. L. Johnston 1952 Further studies on vitamin E deficiency in the lamb. J. Animal Sci., *11:* 609.

Blaxter, K. L., P. S. Watts and W. A. Wood 1952a The nutrition of the young Aryshire calf. 8. Muscular dystrophy in the growing calf. Brit. J. Nutrition, *6:* 125.

Blaxter, K. L., and W. A. Wood 1952b The nutrition of the young Aryshire calf. 9. Composition of the tissues of normal and dystrophic calves. Ibid., 6: 144.

Blaxter, K. L., W. A. Wood and A. M. MacDonald 1953 The nutrition of the young Aryshire calf. 11. The toxicity of cod-liver oil. Ibid., *7:* 34.

Butturini, U. 1949 Clinical and experimental studies on vitamin E. Ann. New York Acad. Sc., 52: 397.

Channon, H. J., and J. A. Buchan Smith 1936 XIX. The dietary prevention of fatty livers. Triethyl-β-hydroxyethylammonium hydroxide. Biochem. J., *30:* 115.

Flipse, R. J., C. F. Huffman, C. W. Duncan and F. Thorpe, Jr. 1948 Potassium versus biotin in the treatment of experimentally induced paralysis in calves. J. Animal Sci., *7:* 525.

Galgan, M. W., M. E. Ensminger and W. L. Slocum 1956 Problems and Practices of Washington Cattlemen. Washington Agr. Exp. Sta. Bull. no. 567, p. 10.

Hove, E. L., and John F. Herndon 1955 Potassium deficiency in the rabbit as a cause of muscular dystrophy. J. Nutrition, *55:* 363.

Hove, E. L., and D. H. Copeland 1954 Progressive muscular dystrophy in rabbits as a result of chronic choline deficiency. Ibid., *53:* 391.

Hubbel, R. B., L. B. Mendel and A. J. Wakemen 1937 A new salt mixture for use in experimental diets. Ibid., *14:* 273.

Johnson, B. C., H. H. Mitchell, J. A. Pinkos and C. C. Morrill 1951 Choline deficiency in the calf. Ibid., *43:* 37.

Keston, A. S., and S. B. Wortis 1946 The antagonistic action of choline and its triethyl analogue. Proc. Soc. Exp. Biol. Med., *61:* 439.

Madsen, L. L., C. M. McKay and L. A. Maynard 1933 Possible relationship between cod-liver oil and muscular degeneration of herbivora fed synthetic diets. Ibid., *30:* 1434.

Melville, R. S., and J. P. Hummel 1951 Creatine and glycocyamine metabolism in rabbits in vitamin E deficiency. J. Biol. Chem., *191:* 383.

Milman, A. E., and A. T. Milhorat 1953 Metabolic patterns in experimentally induced muscular dystrophy. Proc. Soc. Exp. Biol. Med., *84:* 654.

Morgulis, S., and H. C. Spencer 1936a Metabolism studies in nutritional muscular dystrophy. J. Nutrition, *12:* 191.

——— 1936b Studies on the blood and tissues in nutritional muscular dystrophy. Ibid., *12:* 173.

Snedecor, G. W. 1957 Statistical Methods. Iowa State College Press, Ames.

Whiting, F., J. P. Willman and J. K. Loosli 1949 Tocopherol (vitamin E) deficiency among sheep fed natural feeds. J. Animal Sci., *8:* 234.

Willman, J. P., J. K. Loosli, S. A. Asdell, F. B. Morrison and P. Olafson 1946 Vitamin E prevents and cures the "stiff-lamb disease." Cornell Vet., *36:* 200.

The Antithyrotoxic Factor of Liver

V. FAILURE OF THYROTOXICOSIS TO DEPLETE THE ACTIVITY IN SWINE TISSUES

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The defatted, water-insoluble portion of hog liver promotes growth and survival of rats receiving an otherwise lethal amount of thyroxine (Overby et al., '59a). The basal metabolic effects of the thyroxine are not antagonized. It is not clear whether liver provides unidentified vitamin-like entities required for the thyrotoxic rat, or whether crude liver products, rich in protein, merely provide an optimum balance of known nutrients. Excess thyroxine increases the requirement for most vitamins and presumably depletes the body stores of essential nutrients if they are not provided in adequate quantities in the diet.

After Ershoff's ('47) suggestion of an unidentified factor in liver, we did preliminary fractionation studies and found no clear-cut separation of a single active principle. We made the experiments reported herein to help establish the nature of the antithyrotoxic activity in hog liver and to determine whether the activity could be depleted in the tissues of swine by thyrotoxicosis.

EXPERIMENTAL

Swine tissues

Normal liver and muscle were obtained from 10 pigs raised from 50 to 195 pounds with a ration composed of 77.5% of ground yellow corn, 22.5% of protein and trace mineral supplement of the composition shown in table 1. Upon slaughter the entire liver and a portion of the center loin muscle were removed and frozen until used.

Thyrotoxic liver and muscle were obtained from swine fed the above ration plus iodinated casein.[1] One group of 8 males and 2 females averaging 50 pounds in weight received the ration plus 0.1% of iodinated casein for 21 days. The level of iodinated casein was increased to 0.15% for 15 days and then reduced to 0.1% for 26 days because of the excessive toxicity at the higher level. Eight animals survived 51 days and 2 survived 62 days. The average weight gain was 34 pounds. Upon death from thyrotoxicosis, the liver and loin muscle were removed and frozen.

TABLE 1

Composition of swine ration supplement

	%
Soybean meal (44% protein)	47.79
Meat and bone scraps (50% protein)	40.00
Alfalfa meal (17% protein)	10.00
Sodium chloride	2.00
Vitamin and trace mineral mixture[1]	0.21

[1] Composition in grams per kilogram: choline chloride, 26.7; calcium-DL-pantothenate, 26.4; vitamin B_{12}, 0.027; nicotinamide, 24.5; riboflavin, 5.3; viosterol, (1×10^6 IU/gm), 0.64; trace minerals (Blatchford Calf Meal Co., Waukegan, Ill.), 606; zinc oxide, 197; inert ingredients, 113.4.

Another group of 2 males and 3 females, weighing 100 pounds was given the ration with 0.1% of iodinated casein for 77 days and then 0.15% for 30 days. This group tolerated the thyroactive material better than the previous group which started at the 50-pound weight. The second group was sacrificed when weight loss and respiration indicated that death was near.

Preparation of tissues. The livers were ground and extracted twice by slurrying at 90°C with 4 parts of water and filtering

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[1] Protamone, Cerophyl Laboratories, Kansas City, Missouri.

the hot slurry through canvas. The water-insoluble material was dried in air and then extracted with petroleum ether in a Soxhlet extractor. The defatted residue and the fat extract were freed of solvent and reserved for testing in the rat assay.

The ground loin muscle was autoclaved at 120°C for one hour with 200 cc of water per kg of tissue. The melted fat was decanted and discarded. The aqueous slurry was filtered through canvas. The insoluble material was again autoclaved with 500 cc of water per kg of fresh tissue. After filtration, the water-insoluble residue was dried in air and then defatted by continuous extraction with petroleum ether. The characteristics and yields of the various tissues are shown in table 2.

Commercial liver residue[2] was defatted by extracting with petroleum ether.

Defatted beef muscle was purchased commercially.[3]

Rat tests

The assay procedures were reported previously. The basal diet (no. 16) was identical to diet 14 (Overby et al., '59a). The percentage composition was sucrose, 57.5; casein,[4] 30; cottonseed oil, 5; salts (Jones and Foster, '42), 4; cellulose and agar, 3.25; choline chloride, 0.1; and vitamin mixture, 0.15. Groups of 10 male, 21-day old Sprague-Dawley rats received three control rations: the basal diet, the negative control (basal plus 0.35% of iodinated casein) and the standard (negative control plus 10% of defatted commercial liver residue). The test groups (10 rats per group) received the negative-control diet with the test material replacing an equal weight of casein or cottonseed oil. Four- or 5-week growth and survival were the measures of protective activity. Relative activity was referred to the standard by calculating relative potency based on 10% of liver residue as 100 (Overby and Fredrickson, '60).

RESULTS

It was not economically feasible to feed swine a highly-purified ration similar to the rat assay diet. Therefore, it was of interest to establish whether the swine ration used contained antithyrotoxic activity as measured by the rat assay. The swine ration was prepared commercially by mixing ground yellow corn with a high-protein supplement containing vitamins and minerals (table 1). The supplement and corn were tested and showed no significant antithyrotoxic activity at a level of 10% in the rat diet. At 19% of the diet the ground yellow corn was almost completely inactive, but the supplement showed slight activity (table 3). This was expected because of the known activity of the ingredients of the supplement (Overby and Fredrickson, '60). The swine ration was 77.5% corn, therefore the animals received very little "antithyrotoxic activity" in the ration.

The comparative antithyrotoxic activity of tissue fractions prepared from normal and thyrotoxic swine receiving the above ration is shown in table 4. It was obvious that thyrotoxicosis did not deplete the liver and muscle of the unidentified activity. Normal defatted pork liver was 71% as active as the commercial standard, and

[2] Abbott Laboratories, North Chicago, Illinois.
[3] The Wilson Laboratories, Chicago.
[4] Vitamin Test, Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Characteristics of tissues from normal and thyrotoxic animals

Description	Tissue/ body weight	Defatted residue/ fresh tissue	Fat in fresh tissue	Defatted residue		
				Total N	Ash	Moisture
	%	%	%	%	%	%
Normal pork liver	1.34	19.8	4.1	13.65	1.73	5.00
Thyrotoxic pork liver	2.41	18.3	3.5	13.84	2.03	4.56
Commercial liver residue	—	—	—	9.44	13.28	3.09
Normal pork loin	—	13.0	—	14.85	2.17	4.28
Thyrotoxic pork loin	—	13.5	—	14.98	1.62	4.77
Defatted beef muscle	—	—	—	14.44	4.35	4.95

TABLE 3

Antithyrotoxic activity of ground yellow corn and swine ration supplement

Diet treatment	% of diet	5-Week results		Relative potency
		Gain	Survival	
		gm	%	
	Controls			
Basal		231 ± 5.4[1]	100	148
Negative control[2]		117	10	0
Standard[2,3]		178 ± 6.8	90	100
	Test material[2]			
Ground yellow corn	19	123 ± 7.2	30	17
Protein supplement	19	143 ± 7.3	60	50

[1] Standard error $=\sqrt{\Sigma d^2/n\,(n-1)}$.
[2] Finished diet contained 0.35% of iodinated casein.
[3] Defatted commercial liver residue at 10% of the diet.

TABLE 4

Comparative antithyrotoxic activity of tissues from normal and thyrotoxic swine

Diet treatment	% of diet	5-Week results		4-Week results
		Gain	Survival	Relative potency
		gm	%	
	Controls			
Basal		205 ± 5.7[1]	100	152
Negative control[2]		112	10	0
Standards[2,3]	10	156 ± 6.2	90	100
	5	155 ± 5.6	50	68
	Test material[2]			
Normal pork liver residue	10	148 ± 6.1	70	71
Thyrotoxic pork liver residue	10	160 ± 4.4	100	124
Normal pork muscle	10	129 ± 6.8	50	49
Thyrotoxic pork muscle	10	151 ± 13.6	30	63
Normal beef muscle	10	129 ± 19.7	30	38
Normal liver fat	5	115 ± 2.4	30	29
Thyrotoxic liver fat	5	124 ± 5.7	50	39
Commercial liver residue fat	5	115 ± 4.5	90	81

[1] Standard error $=\sqrt{\Sigma d^2/n\,(n-1)}$.
[2] Finished diet contained 0.35% of iodinated casein.
[3] Defatted commercial liver residue at levels indicated.

the thyrotoxic product showed an activity of 124%. On a weight basis, muscle was about one half as active as liver, and again the preparation from thyrotoxic animals was more active. The fat fraction from commercial liver residue was quite active, as previously shown by Overby et al., ('59b). The fats from the test swine were only slightly active, with the fats from thyrotoxic animals showing a slightly higher relative potency. A defatted beef muscle preparation was tested and appeared to have the same order of activity as normal pork muscle.

DISCUSSION

The present experiments would have been most meaningful and interpretable had thyrotoxicosis caused a decrease of antithyrotoxic factor activity in swine tissues. The observed result of an actual increase in activity casts doubt on the presence of any sort of unidentified vitamin-like entity in hog liver. It is difficult to explain why the very tool that creates the demand for the active principles in rats failed to deplete the activity in swine tissues, although this could be due to differences in species. If the presumed fac-

tor were essential for survival of thyrotoxic swine, it should have been reduced to low tissue levels, because the thyrotoxic state was maintained for a long time, and 10 animals were actually allowed to die from thyrotoxicosis. The other 5 proceeded to near death.

The swine ration contained a low level of antithyrotoxic activity when compared with standard liver residue by the rat test. The simultaneous ingestion of the "factor" and the thyroactive compound may have given an increased turnover of "factor" but no net tissue changes. Alternately, the activity may be represented by a hormone or other metabolite produced in swine by endogenous or intestinal synthesis. Up to a point this material could be synthesized to protect the pigs themselves. The synthetic activities may have been increased by the thyroid hormone to meet the excess demand, but the demand was never met because of the continuous ingestion of thyroxine. If the accelerated rate of synthesis continued there could have been no net decrease in the levels of "factor" in tissues. Finally, the protective activity observed in hog liver may be a chance combination of known nutrients in liver not required in increased amount by thyrotoxic swine. Additional experiments are needed before conclusions may be reached.

SUMMARY

Defatted commercial hog liver residue has promoted growth and survival of thyrotoxic rats, an effect not yet reproduced with known nutrients. In this experiment defatted muscle, defatted liver residue and liver fat were prepared from normal swine and swine made thyrotoxic with iodinated casein. In each case the tissue component from the thyrotoxic animals had a higher level of rat protective activity than that from normal swine. In both cases muscle tissue was about one half as active as liver. The results do not support the concept of a vitamin-like entity for the "antithyrotoxic factor."

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LITERATURE CITED

Ershoff, B. H. 1947 Effects of liver feeding on growth and ovarian development in the hyperthyroid rat. Proc. Soc. Exp. Biol. Med., *64:* 500.

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

Overby, L. R., and R. L. Fredrickson 1960 The antithyrotoxic factor of liver. IV. Activity of various pure and crude materials. Ibid., *71:* 129.

Overby, L. R., R. L. Fredrickson and D. V. Frost 1959a The antithyrotoxic factor of liver. I. Method for assay. Ibid., *67:* 397.

Overby, L. R., D. V. Frost and R. L. Fredrickson 1959b The antithyrotoxic factor of liver. II. Comparative activities of defatted liver residue and various fats. Ibid., *68:* 251.

A Study of the Effect of Deoxypyridoxine or Isonicotinic Acid Hydrazide upon Tissue Potassium and Sodium Content of Pyridoxine-Deficient Male Rats[1]

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In previous work in this laboratory Hartsook et al. ('58) observed that decreased potassium retention was associated with the development of a vitamin B_6 deficiency in young male rats. Sodium, but not calcium and magnesium, retention was also reduced significantly as the vitamin B_6 deficiency became more severe. Deoxypyridoxine (DOP) enhanced the reduction of K and Na retention whereas isonicotinic acid hydrazide (INH) significantly increased K and Na retention in rats severely depleted of vitamin B_6.

Hsu et al. ('58) observed that vitamin B_6 deficiency caused an elevation of muscle K and a reduction of muscle Na, but was without effect on K and Na levels in heart, liver and kidneys. These workers found that serum K levels were reduced in vitamin B_6-depleted rats when DOP was injected intraperitoneally at a relatively high level (2.25 mg/rat/week).

Differences in electrolyte retention by various tissues during vitamin B_6 deficiency or the same modified by antagonists should be investigated for theoretical as well as practical reasons. The work of Muntwyler et al. ('53) suggests that a redistribution of tissue K could occur without an alteration in K balance. Studies on the electrolyte concentrations of various tissues might yield information on the mechanisms causing convulsive seizures in vitamin B_6-deficient infants and experimental animals and on the electrolyte metabolism during isoniazid therapy in the treatment of tuberculosis.

The present work reports the concentrations of K and Na in tissues of vitamin B_6-deficient rats and also in combination with dietary DOP and INH under the condition of equal feed intake within groups of animals. The tissues investigated were brain, skeletal muscle, heart muscle, liver, kidneys, blood plasma and blood cells.

EXPERIMENTAL

Twenty-eight male albino rats of the Wistar strain ranging in weight from 32 to 78 gm were allotted to 7 groups of 4 rats each on the basis of body weight and litter. Each group of rats served as a replicate of 4 experimental treatments and the food intake within each group was limited to that of the animal consuming the least. Distilled water was given ad libitum. All animals were placed in individual screen-bottom cages and fed a ground, rat diet[2] until a body weight of 110 to 120 gm was attained. Then the animals were given the experimental diets for periods varying from 34 to 56 days. Rat 1 of each replicate received the basal diet (diet A) having the following percentage composition: vitamin-test casein,[3] 18; sucrose, 35.5; glucose,[4] 30.5; corn oil,[5] 5;

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[2] Rockland Rat Diet, A. E. Staley Manufacturing Company, Decatur, Illinois.

[3] Nutritional Biochemicals Corporation, Cleveland.

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

[5] Oleum percomorphum (Mead-Johnson and Co., Evansville, Indiana) and α-tocopheryl acetate added to the corn oil so that the final diet contained 2,000 I.U. of vitamin A, 283 I.U. of vitamin D, and 10 mg of vitamin E per 100 gm.

mineral mix,[6] 4; vitamin mix,[7] 5; and cellulose, 2. Rats 2, 3 and 4 of each replicate received the basal diet containing per gram, respectively, DOP,[8] 5 μg (diet B); INH,[8] 500 μg (diet C); or pyridoxine·HCl, 10 μg (diet D).

At the end of the feeding period the animals were anesthetized with chloroform, decapitated, exsanguinated, and the following organs immediately excised and freed of extraneous tissues: brain, gastrocnemious muscle, kidneys, liver and heart. The blood was collected directly into test tubes containing approximately 5 mg of lithium oxalate and separated into cell and plasma fractions. These fractions and the excised organs were placed in beakers, dried at 60°C for approximately 30 hours and ashed at 550°C for 24 hours. The ash was extracted with several 20-ml portions of 0.3 N HCl. Potassium and sodium were determined in aliquots of the extracts by flame photometry at $\lambda = 769$ and 589 mμ, respectively. Concentrations of K and Na were expressed as milliequivalents per kilogram of fresh tissue in the case of liver, kidney, brain, skeletal muscle, and heart; as milliequivalents/liter in the case of blood plasma and blood cells.

Analyses of variance were made and the error mean squares were used to compute multiple ranges for testing the statistical significance of differences between treatment means using Duncan's new multiple range test ('55). The error included any inconsistency in the effect of treatment from one age group to another.

RESULTS AND DISCUSSION

Mild symptoms typical of pyridoxine deficiency were observed in animals receiving the vitamin B_6-deficient diets with or without the antagonists, but the deficiencies did not become severe enough for marked inanition or fits to occur.

Data for body weight gains appear in table 1. Gain per day is used because the number of days of experiment differed for the 7 groups of animals: group 1, 49 days; groups 2 and 3, 51 days; groups 4 and 5, 53 days; group 6, 42 days; and group 7, 31 days. In the lower half of the table the treatment means have been arranged in ascending order of magnitude and compared statistically by the multiple range test. Thus, it will be seen that the gains made using diets A and D did not differ significantly and that the gains made with diets B and C were significantly smaller. Gains with diet B were significantly smaller than for diet C.

The data for K and Na content of the tissues appear in table 2. Tissue concentrations of the substances agree, in gen-

[6] Jones and Foster ('42).

[7] Hartsook et al. ('58).

[8] Thanks are due Dr. Laurent Michaud of Merck Sharp and Dohme Research Laboratories Division of Merck and Company, Inc., Rahway, New Jersey for furnishing the deoxypyridoxine and isoniazid used in this study.

TABLE 1

Comparison of body weight gains of moderately vitamin B_6-depleted male albino rats fed semisynthetic diets containing pyridoxine and of rats fed pyridoxine-deficient diets with and without vitamin B_6 antagonists

Item	Treatment means[1]			
	Diet A Basal	Diet B Basal + DOP[2]	Diet C Basal + INH[3]	Diet D Basal + vitamin B_6
Body weight gain gm/day	0.84 ± 0.16[4]	0.15 ± 0.05	0.64 ± 0.24	0.78 ± 0.18
	Comparison of treatment means[5]			
Treatment	B	C	D	A
Mean	0.15	0.64	0.78	0.84

[1] Seven observations per treatment.

[2] Deoxypyridoxine.

[3] Isonicotinic acid hydrazide.

[4] Standard deviation.

[5] Any two means underscored by the same line are not significantly different. Any two means not underscored by the same line are significantly different ($P < 0.05$).

TABLE 2

Tissue Na and K content of moderately vitamin B_6-depleted male albino rats fed semisynthetic diets containing pyridoxine and of rats fed pyridoxine-deficient diets with and without vitamin B_6 antagonists

	Item	Element	Group means[1]			
			Diet A Basal	Diet B Basal + DOP[2]	Diet C Basal + INH[3]	Diet D Basal + vitamin B_6
1	Liver: mEq/kg	K	61.0 ± 8.9[4]	51.0 ± 9.3	53.6 ± 8.6	54.2 ± 12.1
	fresh tissue	Na	57.6 ± 13.7	71.4 ± 12.3	61.7 ± 11.4	64.0 ± 13.9
2	Kidney: mEq/kg	K	54.8 ± 2.7	52.2 ± 4.5	54.1 ± 2.6	50.3 ± 1.6
	fresh tissue	Na	63.4 ± 2.5	67.0 ± 4.7	62.5 ± 4.8	62.8 ± 3.9
3	Brain: mEq/kg	K	81.6 ± 3.4	78.3 ± 6.0	80.1 ± 4.3	80.5 ± 4.4
	fresh tissue	Na	64.7 ± 5.1	67.0 ± 7.4	68.0 ± 6.3	64.7 ± 6.6
4	Muscle: mEq/kg	K	77.8 ± 5.1	75.6 ± 5.3	78.3 ± 3.5	75.8 ± 4.4
	fresh tissue	Na	42.3 ± 6.0	45.7 ± 9.9	39.2 ± 4.6	41.5 ± 9.1
5	Heart: mEq/kg	K	60.2 ± 2.6	61.7 ± 3.3	59.4 ± 1.7	61.6 ± 4.5
	fresh tissue	Na	64.7 ± 10.2	62.4 ± 14.3	66.2 ± 12.9	59.5 ± 8.6
6	Blood plasma:	K	4.1 ± 1.0	4.4 ± 1.8	4.5 ± 1.0	4.6 ± 1.4
	mEq/liter	Na	127.1 ± 8.1	134.3 ± 9.1	128.4 ± 2.4	127.1 ± 5.8
7	Blood cells:	K	52.7 ± 9.8	46.2 ± 13.2	50.0 ± 8.8	54.9 ± 4.0
	mEq/liter	Na	86.7 ± 13.1	94.2 ± 16.9	92.0 ± 14.6	78.6 ± 6.4

[1] Seven observations per group.
[2] Deoxypyridoxine.
[3] Isonicotinic acid hydrazide.
[4] Standard deviation.

eral, very well with those previously reported by Hsu et al., ('58).

Statistical evaluations (identical in type to that used for body weight gain data in table 1) of the data for tissue K and Na content appear in table 3. The Na content of liver, kidney, brain, muscle, and blood plasma of vitamin B_6-deficient rats was either unchanged from, or less than, that of vitamin B_6-adequate animals. The K content of these tissues of vitamin B_6-deficient rats was either unchanged from, or greater than, that of vitamin B_6-adequate animals. On the other hand, although significant differences were not found, the heart and blood cells of vitamin B_6-deficient animals contained greater amounts of Na and lesser amounts of K than those of vitamin B_6-adequate animals.

In many tissues DOP reversed the trend in shift of cation content evident in vitamin B_6 deficiency alone. There were significant increases in the Na content of liver, kidney and plasma (and similar, though nonsignificant, increases in brain, muscle and cells) when DOP was imposed upon vitamin B_6 deficiency (comparison of responses to treatments A and B). There were significant decreases of K content of liver, kidney and brain (and similar, though nonsignificant, decreases in muscle and blood cells) when DOP was imposed upon vitamin B_6 deficiency. The heart response was opposite to that of the other tissues upon DOP treatments—namely, lowered Na content and increased K content—but again the changes were not significant.

A somewhat different pattern of shift of tissue cation content was found when INH rather than DOP was the antagonist imposed upon a vitamin B_6 deficiency. No significant changes in the Na content of tissues were observed due to INH treatment, although nonsignificant increases in the Na content of liver, brain, heart, plasma and cells were observed (comparison of responses to treatments A and C). There was a significant decrease of K content of liver (and similar, though nonsignificant, decreases in kidney, brain, heart and cells) when INH was imposed upon a vitamin B_6 deficiency.

TABLE 3

Comparison of tissue K and Na content of moderately vitamin B_6-depleted male albino rats fed semisynthetic diets containing pyridoxine and of rats fed pyridoxine-deficient diets with and without vitamin B_6 antagonists

	Item		Comparison of treatment means[1]							
			K				Na			
1	Liver: mEq/kg fresh tissue	Treatment	B[2]	C	D	A	A	C	D	B
		Mean	51.0	53.6	54.2	61.0	57.6	61.7	64.0	71.4
2	Kidney: mEq/kg fresh tissue	Treatment	D	B	C	A	C	D	A	B
		Mean	50.3	52.2	54.1	54.8	62.5	62.8	63.4	67.0
3	Brain: mEq/kg fresh tissue	Treatment	B	C	D	A	A	D	B	C
		Mean	78.3	80.1	80.5	81.6	64.7	64.7	67.0	68.0
4	Muscle: mEq/kg fresh tissue	Treatment	B	D	A	C	C	D	A	B
		Mean	75.6	75.8	77.8	78.3	39.2	41.5	42.3	45.7
5	Heart: mEq/kg fresh tissue	Treatment	C	A	D	B	D	B	A	C
		Mean	59.4	60.2	61.6	61.7	59.5	62.4	64.7	66.2
6	Blood plasma: mEq/liter	Treatment	A	B	C	D	A	D	C	B
		Mean	4.1	4.4	4.5	4.6	127.1	127.1	128.4	134.3
7	Blood cells: mEq/liter	Treatment	B	C	A	D	D	A	C	B
		Mean	46.2	50.0	52.7	54.9	78.6	86.7	92.0	94.2

[1] Treatment A indicates basal (−pyridoxine); B indicates basal + 5 μg of deoxypyridoxine (DOP)/gm of diet; C indicates basal + 500 μg of isonicotinic acid hydrazide (INH)/gm of diet; and D indicates basal + 10 μg pyridoxine/gm of diet.

[2] Any two means underscored by the same line are not significantly different. Any two means not underscored by the same line are significantly different ($P < 0.05$).

Although the results obtained in some instances indicate trends only, the differences between treatments would probably reach significant levels in severe vitamin B_6 deficiencies. Severe deficiencies were not imposed. Rather, the animals were brought to a moderate deficiency state so that inanition, which plays an increasingly important part in the physiology of the animal as a deficiency increases in severity, would not seriously limit the food intake of the rats and inject complications of various immeasurable degrees into the interpretation of the data. The ultimate would be attained by accurately measuring tissue electrolyte contents at the point of initiation of convulsions—at this point tissue K would probably be significantly reduced. Some of the differences observed could be due to changes in fat and moisture content of the tissues or, in the case of red blood cells, due to hypochromic microcytic anemia. The possibility that the heart muscle may behave differently than other tissues in its handling of K and Na under the influence of antagonists is most interesting. A tendency towards a differential response in the shift of heart K and Na content to DOP and INH treatment may be of a nature similar to the differential response of liver enzymes to antagonist and/or deficiency thereapy noted by Dietrich and Shapiro ('53) and by Hartsook et al. ('58).

SUMMARY

Seven groups of 4 rats each were fed a pyridoxine-free basal diet or the basal diet plus either deoxypyridoxine (DOP) isonicotinic acid hydrazide (INH) or pyridoxine. Based upon K and Na assays of liver, kidney, brain, muscle, heart, blood plasma and blood cells, the following observations were made:

When vitamin B_6-deficient animals were compared with vitamin B_6-adequate animals, Na content remained constant or decreased and K content remained constant or increased in all tissues studied except heart and blood cells, where a tendency toward opposite effects was noted.

When DOP-treated vitamin B_6-deficient animals were compared with vitamin B_6-deficient animals, Na content remained constant or increased and K content remained constant or decreased in all tissues except heart and blood plasma, where a tendency toward opposite effects was noted.

When INH-treated vitamin B_6-deficient animals were compared with vitamin B_6-deficient animals, Na content remained constant or increased and K content remained constant or decreased in all tissues except kidney, muscle and blood plasma and blood cells where a tendency toward opposite effects was noted.

LITERATURE CITED

Dietrich, L. S., and D. M. Shapiro 1953 Antagonist studies: Comparison of desoxypyridoxine and pyridoxine-deficiency therapy on various vitamin B_6 enzymes. Proc. Soc. Exp. Biol. Med., *84:* 555.

Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.

Hartsook, E. W., T. V. Hershberger and C. E. French 1958 A study of the effect of deoxypyridoxine or isoniazid upon mineral retention and liver enzyme activities of pyridoxine-deficient male rats. J. Nutrition, *65:* 547.

Hsu, J. M., R. L. Davis and B. F. Chow 1958 Electrolyte imbalance in vitamin B_6-deficient rats. J. Biol. Chem., *230:* 889.

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

Muntwyler, E., G. E. Griffin and R. L. Arends 1953 Muscle electrolyte composition and balances of nitrogen and potassium in potassium-deficient rats. Am. J. Physiol., *174:* 283.

Amino Acid Balance and Imbalance

V. EFFECT OF AN AMINO ACID IMBALANCE INVOLVING NIACIN ON LIVER PYRIDINE NUCLEOTIDE CONCENTRATION IN THE RAT[1]

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It is well known that rats fed a niacin-deficient diet in which the only source of protein is 9% of casein grow slowly but otherwise appear normal; and that if gelatin or some other tryptophan-deficient protein or threonine is added to this diet, growth is retarded and the animals develop what appears to be a niacin deficiency (Krehl et al., '46; Hankes et al., '48). The growth retardation is attributed to an amino acid imbalance involving niacin and tryptophan because the addition of either niacin or tryptophan to these diets prevents the development of the deficiency signs and stimulates growth. The ability of the rat to convert tryptophan to niacin has made it difficult to resolve the question of whether the substances causing such imbalances affect tryptophan utilization, niacin utilization or the formation of niacin from tryptophan (Henderson et al., '53; Salmon, '54); and also the question of the extent to which the deficiency signs are directly attributable to niacin deficiency.

Since diets used to study amino acid imbalances involving niacin and tryptophan lack niacin and are low in tryptophan, the niacin stores of rats fed such diets should be low; and one might expect that the concentrations of pyridine nucleotides in their tissues would be low, particularly if the imbalance inhibits niacin utilization or the conversion of tryptophan to niacin. With this as a working hypothesis, a study has been made of the effect of an apparent niacin deficiency induced by an amino acid imbalance on liver pyridine nucleotide concentrations in the rat.

EXPERIMENTAL

Male weanling rats of the Holtzman strain weighing 40 to 50 gm were used in all but one of these experiments. In that one, indicated in the tables, Rolfsmeyer strain rats were used. These appear similar to the Holtzman strain but in our experience grow somewhat more slowly. The rats were housed in individual suspended cages and fed the basal diet for two to three days to allow them to adapt to the environment. They were then separated into groups of 5 rats each so that the average weights of the groups did not differ by more than one gram. The groups of rats were fed ad libitum the experimental diets indicated in the tables of results, and weighed three times weekly during the two-week experimental period. The percentage composition of the basal diet was as follows: casein, 8.0 or 9.0; DL-methionine, 0.3; salt mixture, 5.0 (Harper, '59);

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corn oil containing fat-soluble vitamins, 5.0; water-soluble vitamins (less niacin) in sucrose, 0.25 (Harper, '59); choline chloride, 0.15; and sucrose to make 100. The other components in the experimental diets replaced an equal weight of sucrose.

At the end of the two-week experimental period, the animals were killed by a blow on the head, decapitated and their livers removed and analyzed for pyridine nucleotides. Oxidized pyridine nucleotides were determined by the method of Feigelson et al., ('50), which involved homogenization of the liver in trichloracetic acid, adsorption of the extracted pyridine nucleotides on charcoal, elution with pyridine, and spectrophotometric analysis of the eluate at 340 mμ after reduction with hydrosulfite. Reduced pyridine nucleotides were measured by a modification of the method of Lowry et al., ('57). The reduced pyridine nucleotides were extracted in hot Na_2CO_3 and the fluorescence was measured in a photofluorometer after treatment of the extract with strong NaOH and H_2O_2.

RESULTS

The results presented in table 1 show that liver pyridine nucleotide concentrations of rats fed basal diets containing either 8 or 9% of casein were the same, and that the addition of 0.36% of DL-threonine, which caused a retardation of the growth of rats fed the 8% casein diet, did not depress pyridine nucleotide concentrations. Even the addition of gelatin, which severely retarded the growth of rats fed diets containing either 8 or 9% of casein, did not cause any lowering of liver pyridine nucleotide concentration.

When tryptophan was added to either basal diet, no growth response occurred, but pyridine nucleotide concentration increased. Pyridine nucleotide concentration, as well as growth, increased when tryptophan was added with threonine, the increase being 150 to 200 μg/gm of liver. On the other hand, addition of tryptophan to diets containing gelatin did not cause a significant rise in pyridine nucleotide concentration, but growth was greatly increased.

Addition of 2.5 mg of niacin per 100 gm of diet which stimulated the growth of groups receiving threonine or gelatin, caused no consistent increase in liver pyridine nucleotide concentration under any condition. In the groups in which there was an apparent response, the standard errors were very large. Addition of niacin with tryptophan did not increase liver pyridine nucleotide concentrations above values obtained with tryptophan alone.

Concentrations of reduced liver pyridine nucleotides were determined in all cases

TABLE 1

Effect of niacin and tryptophan on liver pyridine nucleotide (P.N.) concentrations of rats fed a low casein diet with added gelatin or DL-*threonine*[1]

Supplements				9% Casein		8% Casein[2]	
Gelatin	DL-Threonine	Nicotinic acid	L-Tryptophan	Gain in weight	Oxidized liver P.N.	Gain in weight	Oxidized liver P.N.
%	%	*mg/100 gm*	%	*gm/2 weeks*	*μg/gm*	*gm/2 weeks*	*μg/gm*
—	—	—	—	35	638 ± 7[3]	15	636 ± 84
—	—	2.5	—	43	657 ± 32	13	712 ± 107
—	—	—	0.1	40	755 ± 45	13	851 ± 101
—	—	2.5	0.1			14	842 ± 94
—	0.36	—	—	30	688 ± 50	4	725 ± 75
—	0.36	2.5	—	48	688 ± 14	35	819 ± 124
—	0.36	—	0.1	50	822 ± 57	32	1017 ± 128
—	0.36	2.5	0.1	45	874 ± 44	39	929 ± 69
6.0	—	—	—	6	679 ± 34	5	596 ± 50
6.0	—	2.5	—	52	791 ± 49	27	532 ± 10
6.0	—	—	0.1	62	729 ± 25	32	627 ± 7
6.0	—	2.5	0.1	60	749 ± 21	33	617 ± 45

[1] All diets contained 0.3% of DL-methionine.
[2] Rolfsmeyer strain rats.
[3] Standard error of the mean.

but are not shown in the tables because they were affected very little by the various dietary changes. Reduced pyridine nucleotide values for the groups of rats fed the basal, threonine- and gelatin-supplemented diets were, respectively, 235 ± 21, 272 ± 32 and 293 ± 12 μg/gm liver. Values for the corresponding groups that also received tryptophan, which increased oxidized pyridine nucleotide concentrations, were 216 ± 29, 282 ± 36 and 259 ± 11 μg/gm, respectively.

Since amino acid imbalances which increased the severity of deficiency signs caused no depression in liver pyridine nucleotide concentration in two weeks, a longer experimental period was tried. Rats were maintained on diets containing 8% of casein and 6% of gelatin with or without niacin for 10 weeks. The growth and pyridine nucleotide values for these groups are shown in table 2. Rats fed the niacin-free diets grew very little, were emaciated, and appeared to have developed a severe niacin deficiency, but again the pyridine nucleotide concentrations were the same whether niacin was present in the diet or not.

Although the liver pyridine nucleotide concentration of rats receiving no niacin in the diet for 10 weeks had not changed, it was considered possible that the ability of the animals to synthesize pyridine nucleotides might be affected. To test this, rats from the groups maintained for 10 weeks on the niacin-free or the niacin-supplemented diet were injected intraperitoneally with nicotinamide, 50 mg/100 gm of body weight, and the concentrations of pyridine nucleotides in their livers were measured after 8 hours. Kaplan et al. ('56) had shown that the injection of nicotinamide stimulated pyridine nucleotide synthesis in the liver, with the concentration being near maximal 8 to 12 hours after injection. The results shown in table 3 indicated that the ability of the livers of rats fed the niacin-free diet containing gelatin, to synthesize pyridine nucleotides following an injection of nicotinamide was not impaired — the average pyridine nucleotide concentration being about three times that of the uninjected control group, a response comparable to that obtained in rats fed an adequate diet. There was also a rise in the pyridine nucleotide concentration of the liver in non-deficient rats injected with nicotinamide, but the average increase was less than that for the deficient rats. As the concentration was measured at only one time interval, no significance can be attached to this observation.

As the addition of 6% of gelatin to the basal diet caused a severe retardation of

TABLE 2

Concentration of liver pyridine nucleotides of rats fed for 10 weeks an 8% casein-6% gelatin diet lacking niacin

Supplement[1]	Final weight	Liver pyridine nucleotides	
		Oxidized	Reduced
	gm	*μg/gm*	*μg/gm*
None	77	737	298
2.5 mg nicotinic acid/100 gm diet	210	776	223

[1] Both diets contained 0.3% of DL-methionine.

TABLE 3

Effect of injection of nicotinamide on liver pyridine nucleotide concentration in rats fed for 10 weeks diets containing 8% casein and 6% gelatin

Dietary supplement	Liver pyridine nucleotides (oxidized)	
	Uninjected controls	8 hours after injection[1]
	μg/gm	*μg/gm*
None	737	2430
2.5 mg nicotinic acid/100 gm diet	776	1650

[1] Fifty milligrams of nicotinamide/100 gm body weight injected intraperitoneally.

growth in both the two-week and 10-week experimental periods but no fall in liver pyridine nucleotide concentration, the effect of a more severe imbalance caused by adding 12% of gelatin to the basal diet was determined. In addition, since in the studies described above niacin had not been effective in increasing the liver pyridine nucleotide concentration, the effect of adding an equivalent quantity of nicotinamide was determined. The results presented in table 4 show that even a severe growth depression, which could be only partially prevented by niacin alone, did not cause a decline in liver pyridine nucleotide concentration. Nicotinamide was no more effective than nicotinic acid, either at 2.5 or 5.0 mg/100 gm of the diet, in increasing the concentration of liver pyridine nucleotides of rats fed diets containing 6 or 12% of gelatin. Nor did tryptophan, which was effective in restoring growth regardless of the level of gelatin in the diet, cause liver pyridine nucleotide concentrations to rise. There appears to be a trend toward higher values in this experiment in the groups receiving the higher percentage of gelatin, but this was not consistenly true. The one particularly high value of 758 μg/gm seems to be anomalous.

There was a marked increase in the concentration of liver pyridine nucleotides when the diet containing threonine was supplemented with tryptophan, but only a slight and variable increase when tryptophan was added to the diet containing gelatin, yet the growth rate increased in both cases. As Lyman and Elvehjem ('51) produced imbalances by adding mixtures of amino acids to diets containing 9% of casein, certain combinations of amino acids were tested for their ability to prevent a rise in the concentration of pyridine nucleotides when tryptophan was added to the diet. Groups of animals were fed for two weeks diets to which amino acid mixtures causing imbalances had been added, then they were given supplements of niacin (5.0 mg/100 gm of diet) or tryptophan (0.2%) for 10 days. The results of the liver pyridine nucleotide analyses at the end of this time are shown in table 5. Niacin stimulated the growth of the rats but had no effect on liver pyridine nucleotide concentrations. The addition of 1. 89% of amino acids containing 0.36% of DL-threonine to the basal diet did not prevent the increase in liver pyridine nucleotide concentration caused by additional tryptophan.

DISCUSSION

Growth retardations resulting from amino acid imbalances caused by adding threonine or gelatin to diets low in niacin or tryptophan have been attributed to niacin deficiency due (1) to a depressed rate of conversion of tryptophan to niacin (Rosen and Perlzweig, '49; Henderson et

TABLE 4

Effect of nicotinic acid, nicotinamide and tryptophan on liver pyridine nucleotide concentrations of rats fed diets containing 8% of casein and 6% or 12% of gelatin

Dietary supplements[1]				Gain in weight	Oxidized liver pyridine nucleotides
Gelatin	Nicotinic acid	Nicotinamide	L-Tryptophan		
%	*mg/100 gm*	*mg/100 gm*	*%*	*gm/2 weeks*	*μg/gm*
—	—	—	—	36.0	548 ± 40[2]
6.0	—	—	—	7.8	518 ± 28
6.0	2.5	—	—	36.8	601 ± 16
6.0	—	2.5	—	42.0	567 ± 19
6.0	—	—	0.1	55.8	607 ± 26
12.0	—	—	—	5.2	662 ± 136
12.0	2.5	—	—	29.4	619 ± 24
12.0	2.5	—	0.1	59.6	758 ± 59
12.0	2.5	2.5	—	24.0	653 ± 34
12.0	5.0	—	—	22.8	640 ± 61
12.0	—	5.0	—	24.2	661 ± 80
12.0	—	2.5	0.1	67.0	650 ± 64

[1] All diets contained 0.3% of DL-methionine.
[2] Standard error of the mean.

TABLE 5

Effect of the addition of nicotinic acid or tryptophan to the diets of rats which had been fed for two weeks imbalance diets containing threonine and amino acid mixtures

	Supplements[1]	Gain in weight		Oxidized liver pyridine nucleotides
		For 2 weeks before supplementation	10 days following supplementation	
		gm	*gm*	*μg/gm*
1	1.26% AA mix 1[2] + 5.0 mg nicotinic acid/100 gm diet	9.0	44.0	512
2	1.26% AA mix 1 + 0.2% L-tryptophan		47.0	988
3	1.89% AA mix 1 + 5.0 mg nicotinic acid/100 gm diet	11.5	48.4	642
4	1.89% AA mix 1 + 0.2% L-tryptophan		51.6	1147

[1] All diets contained 0.3% of DL-methionine and 0.36% of DL-threonine.

[2] AA mix 1 indicates amino acid mixture 1 (0.26 gm of DL-phenylalanine; 0.3 gm of L-lysine·HCl, 0.07 gm of L-histidine·HCl); 0.63 gm is the quantity in 6% of gelatin.

al., '53; Koeppe and Henderson, '55) or (2) to a reduction in the efficiency of utilization of tryptophan generally, with niacin deficiency occurring only secondarily to this (Salmon, '54). A review of the evidence bearing on these hypotheses has led us to conclude that imbalances involving niacin and tryptophan are typical amino acid imbalances for which no special explanation is necessary and that niacin deficiency is involved only indirectly.

The following observations support this conclusion. (1) If the primary deficiency were that of niacin, niacin should be more effective than tryptophan in overcoming the growth-retarding action of gelatin. However, tryptophan is more effective than niacin in overcoming the imbalance created by adding a large amount of gelatin to the diet (Salmon, '54; Harper and Kumta, '59). (2) That a niacin deficiency develops because tryptophan is used to a greater extent for protein synthesis (Henderson et al., '53) is unlikely because the imbalance causes growth retardation and, therefore, less synthesis of new tissue. This could conceivably result in more, rather than less, tryptophan becoming available for niacin synthesis. (3) The possibility that the imbalance causes a reduction in the concentration of tissue pyridine nucleotides which in turn results in a growth depression by limiting the rate of conversion of tryptophan to niacin (Koeppe and Henderson, '55) is also unlikely for in none of the present experiments were liver pyridine nucleotide concentrations of rats fed diets in which an imbalance has been caused by the addition of threonine or gelatin lower than those of control rats (table 4). This observation also implies that some tryptophan is converted to niacin even when the imbalance is severe (table 4). Brown et al., ('58) found that human subjects, in negative nitrogen balance due to the ingestion of a tryptophan-deficient diet, continued to excrete metabolites that arise during the conversion of tryptophan to niacin. (4) The pathway from tryptophan to niacin is operative in rats fed a diet containing sufficient threonine to cause an imbalance because their liver pyridine nucleotide concentrations rise in response to a supplement of tryptophan (Shahinian et al., '52; table 1). Also if the tryptophan intake of rats fed a diet in which the imbalance is caused by gelatin is sufficiently high, N-methyl nicotinamide excretion increases (Rosen and Perlzweig, '49) and so does liver pyridine nucleotide concentration.[3] The failure, in the present study, of a relatively small supplement of tryptophan to cause a rise in the liver pyridine nucleotide concentrations of rats receiving gelatin may have been a consequence of the increased growth rate of the animals. Chaloupka et al. ('57) have shown that tryptophan is used first for

[3] Unpublished results.

protein synthesis and only secondly for the synthesis of pyridine nucleotides in rats depleted of niacin and tryptophan. (5) Vitamin deficiencies studied in this laboratory do not result in depressions in growth rate and food intake of normal weanling rats until after the animals have been fed the vitamin-deficient diet for over one week, particularly if the diet is low in protein. These effects occur within one to three days when gelatin or an amino acid mixture containing threonine is used to cause an imbalance involving niacin and tryptophan. This is characteristic of many amino acid imbalances and amino acid deficiencies.

The one observation that appears to be somewhat inconsistent with this conclusion is that niacin supplementation alone prevented the growth retardation caused by most of these imbalances but did not stimulate pyridine nucleotide synthesis. However, Oesterling and Rose ('52) described a tryptophan-sparing effect of niacin in growth studies which has been confirmed in our laboratory. If the initial niacin supplement were used instead of tryptophan to maintain tissue pyridine nucleotide concentrations, more tryptophan would be available for protein synthesis, and, unless the niacin supplement was very large, little rise in the concentration of liver pyridine nucleotides would be expected. Thus, this observation on the effect of niacin would not be at variance with the conclusion that growth retardation caused by the imbalance is primarily due to an increase in the severity of the deficiency of tryptophan.

The sparing effect of niacin on tryptophan should be investigated in more detail, because it implies that there is a feed-back mechanism by which a dietary supply of niacin inhibits the conversion of tryptophan to niacin so that more tryptophan is made available for protein synthesis.

Finally, are diets in which a combined tryptophan and niacin deficiency is induced by an amino acid imbalance suitable for investigations of the relative effectiveness of niacin and tryptophan as precursors of blood and liver pyridine nucleotides in the rat? Feigelson et al., ('51a, b) and Williams et al., ('50, '51) reported that niacin and tryptophan are equally effective as precursors of liver pyridine nucleotides if they are added to the diet in equimolar quantities, and that tryptophan is a more effective precursor if the quantities of niacin and tryptophan fed are in the range of the requirements. Duncan and Sarett ('51) questioned the validity of these conclusions because Feigelson et al., and Williams et al., used diets that were devoid of either protein or tryptophan and thus niacin was tested in animals unable to synthesize protein. Sarett and Perlzweig ('43) had previously reported that the concentration of niacin in the liver of the rat varied with the protein intake and was independent of niacin intake. They suggested, therefore, that the incorporation of niacin into the liver must be accompanied by protein deposition. As diets of the type used in the present study are low in both niacin and tryptophan, yet permit protein synthesis (growth) when either niacin or tryptophan is added, they offer promise for studies designed to resolve the question raised by Duncan and Sarett. However, since liver pyridine nucleotide concentrations of rats fed imbalanced diets containing 8% of casein do not fall below those of normal rats, a diet with a still lower casein content would appear necessary in order to decrease still further the amount of tryptophan available for conversion to niacin.

SUMMARY

The effects of an amino acid imbalance on growth and liver pyridine nucleotide concentrations of rats fed niacin-deficient diets containing 8% of casein have been determined. Growth depressions caused by adding threonine or 6% of gelatin to the diet were prevented by niacin supplementation, but those caused by adding 12% of gelatin were not. Liver pyridine nucleotide concentrations were not low even when the growth depression was severe and were unaffected by niacin supplementation.

The growth depressions caused by threonine or gelatin were prevented by supplementation of the diet with tryptophan. A supplement of tryptophan caused a rise in liver pyridine nucleotide concen-

tration when the imbalance was caused by threonine, but not when it was caused by gelatin.

The conclusion has been drawn from consideration of these results and those of other investigators that imbalances involving niacin and tryptophan are true amino acid imbalances comparable with those demonstrated in diets not deficient in niacin, and that the involvement of niacin is secondary.

LITERATURE CITED

Brown, R. R., J. M. Vivian, M. S. Reynolds and J. M. Price 1958 Some aspects of tryptophan metabolism in human subjects. II. Urinary tryptophan metabolites on a low niacin diet. J. Nutrition, *66:* 599.

Chaloupka, M. M., J. N. Williams, Jr., M. S. Reynolds and C. A. Elvehjem 1957 Relative roles of niacin and tryptophan in maintaining blood pyridine nucleotides, nitrogen balance and growth in adult rats. Ibid., *63:* 361.

Duncan, M., and H. P. Sarett 1951 Effect of nicotinic acid and tryptophan on pyridine nucleotides in red blood cells in man. J. Biol. Chem., *193:* 317.

Feigelson, P., J. N. Williams, Jr. and C. A. Elvehjem 1950 Spectrophotometric estimation of pyridine nucleotides in animal tissues. Ibid., *185:* 741.

——— 1951a Pyridine nucleotide formation from tryptophan and niacin. Ibid., *192:* 737.

——— 1951b Comparative effect of tryptophan, niacin, and nicotinamide in forming liver pyridine nucleotides. Proc. Soc. Exp. Biol. Med., *78:* 34.

Hankes, L. V., L. M. Henderson, W. L. Brickson and C. A. Elvehjem 1948 Effect of amino acids on the growth of rats on niacin-tryptophan deficient rations. J. Biol. Chem., *174:* 873.

Harper, A. E. 1959 Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance. J. Nutrition, *68:* 405.

Harper, A. E., and U. S. Kumta 1959 Amino acid balance and protein requirement. Federation Proc., *18:* 1136.

Henderson, L. M., O. J. Koeppe and H. H. Zimmerman 1953 Niacin-tryptophan deficiency resulting from amino acid imbalance in non-casein diets. J. Biol. Chem., *201:* 697.

Kaplan, N. O., A. Goldin, S. R. Humphreys, M. M. Giotto and F. E. Stolzenbach 1956 Pyridine nucleotide synthesis in the mouse. Ibid., *219:* 287.

Koeppe, O. E., and L. M. Henderson 1955 Niacin-tryptophan deficiency resulting from imbalances in amino acid diets. J. Nutrition, *55:* 23.

Krehl, W. A., P. S. Sarma and C. A. Elvehjem 1946 The effect of protein on the nicotinic acid and tryptophan requirement of the growing rat. J. Biol. Chem., *162:* 403.

Lowry, O. H., N. R. Roberts and J. I. Kapphahan 1957 The fluorometric measurement of pyridine nucleotides. Ibid., *224:* 1047.

Lyman, R. L., and C. A. Elvehjem 1951 Further studies on amino acid imbalance produced by gelatin in rats on niacin-tryptophan low ration. J. Nutrition, *45:* 101.

Oesterling, M. J., and W. C. Rose 1952 Tryptophan requirement for growth and utilization of its optical isomers. J. Biol. Chem., *196:* 33.

Rosen, F., and W. A. Perlzweig 1949 The effect of gelatin in the transformation of tryptophan to niacin in rats on low casein diets. Ibid., *177:* 163.

Salmon, W D. 1954 Tryptophan requirement of the rat as affected by niacin and level of dietary nitrogen. Arch. Biochem. Biophys., *51:* 30.

Sarett, H. P., and W. A. Perlzweig 1943 Effect of protein and B vitamin levels of the diet upon the tissue content and balance of riboflavin and nicotinic acid in rats. J. Nutrition, *25:* 173.

Shahinian, S. S., K. Ebisuzaki, J. P. Kring, J. N. Williams, Jr. and C. A. Elvehjem 1952 The action of threonine in inducing an amino acid imbalance. Proc. Soc. Exp. Biol. Med., *80:* 146.

Williams, J. N., Jr., P. Feigelson and C. A. Elvehjem 1950 Relation of tryptophan and niacin to pyridine nucleotides of tissue. J. Biol. Chem., *187:* 597.

Williams, J. N., Jr., P. Feigelson, S. S. Shahinian and C. A. Elvehjem 1951 Further studies on tryptophan-niacin-pyridine nucleotide relationships. Ibid., *189:* 659.

Relationship Between Metabolism of Xylose and Cataractogenesis in the Weanling Rat[1]

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The mechanism whereby xylose-induced cataractous changes are produced has not been established. One curious feature of the lenticular response to this sugar, but not to galactose, is its reversibility (Darby and Day, '40). In susceptible strains of rats the characteristic equatorial vacuoles appear in the lens after 5 days of xylose feeding, increase both in size and in the extent of the lens involved but begin to regress between days 12 and 15. By the 21st day, only a few peripheral vacuoles remain and in susceptible strains, only 10% of the animals develop lenticular opacities. During the first 12 days, when changes are progressive, there is a loss of lens potassium characteristic also of galactose-induced cataractogenesis. With xylose, however, lens potassium reaccumulates as lenticular changes regress.

The unique susceptibility of the lens of young but not older rats and the mechanism whereby reversal of cataractous changes occurs is not known. One factor could be a decrease in the xylose load presented to the lens at the time regression of lens changes occurs. Another possibility is a change in the susceptibility of the lens itself even in the presence of continued high concentrations of xylose. In support of this second hypothesis is the finding of Van Heyningen ('59) that xylitol and sorbitol accumulate in the lens of the young susceptible rat and are also present in the older animals which are resistant to the cataractogenic effects of xylose. It has been shown that weanling rats fed high-carbohydrate diets show a transient self-limiting rise in metabolic rate.[2] This calorigenic response occurs at the period of greatest susceptibility to cataractogenesis. As high-carbohydrate diets are usually employed in studies of xylose-induced cataractogenesis, this elevation in oxygen consumption may play an essential or permissive role in the age-dependent change in susceptibility. This calorigenic response can be abolished by feeding weanling animals sufficient high-carbohydrate diet to just permit maintenance of body weight for several days before ad limitum feeding is started. This process is termed "adaptation." The role of the rise in metabolism on cataractogenesis may be assessed by following lenticular changes in animals started on the xylose at weaning and in such adapted rats fed diets containing xylose.

In these studies the magnitude of the xylose load during appearance and regression of cataractous changes was studied by balance techniques. In addition blood xylose was determined during this experimental period. Lens changes were also studied in weanling rats fed the diet at weaning and in animals adapted to the diet so as to eliminate the calorigenic response associated with high carbohydrate intake in weanling animals.

METHODS

Weanling male Holtzman strain rats were housed in a room held at constant temperature. Food and water were allowed ad libitum. The diet consisted (in per cent) of glucose, 30; xylose, 30; casein, 21; fat,[3] 15; salt, 4; and a complete vitamin supplement. Control animals received the same diet with glucose replacing the xy-

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[2] Heggeness, F. W. 1960 Calorigenic response to high carbohydrate intake in weanling rats. Federation Proc., *19:* 324 (abstract).
[3] Crisco.

lose present in the experimental diet. The NaCl content of the diets was 1.3%. Oxygen consumption determinations were carried out on the control glucose- and xylose-fed animals by the method of Watts and Gourley ('53).

For the balance studies, a group of animals was housed in stainless steel metabolism cages. Food and water were allowed ad libitum but amounts ingested were measured at three-day intervals. Urine was collected in dilute sulfuric acid under toluene. Three-day collections were pooled and urinary xylose excretion determined by the method of Tracey ('50). Blood xylose was similarly determined in other animals ingesting the same diet. Lens potassium was measured by flame photometry following ashing of lens at 500°C.

Animals were adapted to the high-carbohydrate diet by the daily feeding of 3 gm of a 60% glucose diet for three days. The 30% xylose diet was started after such a period of adaptation.

RESULTS

Balance studies. In table 1 are shown the results of the balance studies on xylose-fed weanling rats. All animals fed a 30% xylose diet had polyuria and polydipsia. Water intake averaged 13 ml per day during the first three-day period and gradually increased to 35 ml per day between days 18 to 21. Diarrhea was also present for at least the first 12 days of feeding. At autopsy the gastrointestinal tracts of xylose-fed animals were found to be distended with gas. Xylose intake was initially approximately 1 gm per day but increased gradually, and from the 9th day about 3 gm per day were ingested. Urinary xylose excretion during these three-day balance studies paralleled intake but did not exceed 30% of the ingested load.

Urinary xylose excretion per 100 gm of animal remained relatively low during the first two three-day periods and then increased to and remained relatively constant at approximately 2.5 gm per 100 gm of body weight. On the third day of feeding, blood xylose concentration was 20.5 ± 2.9 mg/100 ml. On days 6, 9 and 12, it was 32.2 ± 4.9, 27.1 ± 1.4, and 28.4 ± 2.5 mg/100 ml, respectively. On the 18th day, when regression of lenticular changes was well underway, blood xylose was 30.2 ± 2.2 mg/100 ml.

Calorigenic response. The calorigenic response found to be associated with the feeding of a 60% glucose diet was relatively unchanged by the replacement of up to 50% of the hexose with xylose (table 2). The slower response of xylose-fed animals as compared with those fed glucose appears to be due to an initial voluntary restriction of intake when diets containing xylose are fed. After the 9th day, oxygen consumptions remained at about 1 l/hour/$kg^{3/4}$.

In weanling rats fed 3 gm of the 60% glucose diet for three days, followed by the 30% xylose diet, food intake, growth rate and efficiency of utilization were significantly greater than in the animals fed the xylose diet immediately (table 3). In such adapted animals, the typical equatorial vacuolization characteristic of early cataract formation was first observed on day

TABLE 1

Xylose intake and urinary excretion in ad libitum-fed weanling rats[1]

Days	Xylose intake	Urinary xylose excretion	Xylose excreted
	gm/3 days	*gm/3 days*	*gm/100 gm body weight/ 3 days*
1–3	3.31 ± 0.08[2]	0.85 ± 0.15	1.72 ± 0.33
4–6	4.74 ± 0.80	0.68 ± 0.06	1.25 ± 0.11
7–9	5.11 ± 0.28	1.60 ± 0.12	2.44 ± 0.25
10–12	7.48 ± 0.66	2.36 ± 0.34	3.04 ± 0.45
13–15	10.20 ± 0.51	2.32 ± 0.16	2.48 ± 0.18
16–18	8.80 ± 0.36	2.53 ± 0.21	2.46 ± 0.20
17–21	9.34 ± 0.38	3.06 ± 0.26	2.85 ± 0.20

[1] Twelve animals.
[2] Standard error of the mean.

TABLE 2

Oxygen consumption of weanling rats in liters/hour/kilogram$^{3/4}$ fed the xylose and glucose diets[1]

Day	Xylose diet	Glucose diet
0	0.90 ± 0.03[2]	0.90 ± 0.03
1	1.05 ± 0.03	1.31 ± 0.05
3	1.18 ± 0.05	1.26 ± 0.06
5	1.43 ± 0.03	1.14 ± 0.06
7	1.37 ± 0.04	1.03 ± 0.03
9	1.13 ± 0.04	1.10 ± 0.06

[1] Each group contained 7 animals.
[2] Standard error of the mean.

4 and not on day 5 as in animals fed the xylose diet from the beginning. The lens changes progressed rapidly, and more extensive involvement of the lens developed than in the animals fed xylose at weaning. The loss of lens potassium was also significantly greater. Regression of lenticular vacuoles was first observed on day 12 as in the unadapted animals. Significant reaccumulation of lens potassium was observed by day 15. Too few animals received the diet for a sufficient period of time to determine whether incidence of cataract formation was also greater in the adapted group. The abolishment of the calorigenic response appears to aggravate rather than lessen the severity of the lenticular changes.

DISCUSSION

Xylose is utilized to a very limited extent in the rat (Miller and Lewis, '32). Hiatt ('57) found only 1% of injected labeled xylose to be incorporated into glycogen. In the studies reported here, 60 to 75% of the ingested xylose was not recovered. The non-recovered fraction remained relatively constant and no increase in metabolized plus destroyed xylose developed. It is likely that this great difference between intake and urinary recovery is largely due to destruction of this sugar by intestinal bacteria and by fecal losses. Reversal of lens changes occurs in the presence of a relatively constant xylose load.

The particular susceptibility of the weanling rat to cataractogenesis also appears not to depend upon the calorigenic response associated with the feeding of the high-carbohydrate diets. Lenticular changes are even more severe when this metabolic response is absent. In support of the finding that the calorigenic re-

TABLE 3

Food intake, weight gains and lens K^+ in adapted and non-adapted animals fed 30% xylose diets[1]

Day	Body weight		Food intake/3 days		Lens K^+ mEq/gm weight	
	Adapted	Unadapted	Adapted	Unadapted	Adapted	Unadapted
	gm	*gm*	*gm*	*gm*		
0	46.3 ± 6.0[2]	50.2 ± 1.2			50.6 ± 4.1	50.6 ± 4.1
3	51.8 ± 1.1	50.5 ± 1.4	16.3 ± 0.8	11.0 ± 0.7		
6	63.4 ± 1.0	58.7 ± 1.8	19.8 ± 0.3	16.3 ± 0.8		47.3 ± 4.4
9	78.0 ± 1.3	72.0 ± 1.7	26.3 ± 0.6	17.2 ± 1.0	13.0 ± 2.1	36.0 ± 4.4
12	92.7 ± 2.1	83.2 ± 2.4	26.3 ± 0.6	25.0 ± 1.4	7.6 ± 1.1	50.1 ± 4.4
15	106.5 ± 1.2	99.4 ± 3.2	31.3 ± 1.9	33.7 ± 1.7	25.7 ± 1.9	42.2 ± 5.1
18	113.0 ± 1.3	111.6 ± 3.6	27.0 ± 1.3	29.9 ± 1.3	32.2 ± 0.1	54.2 ± 5.1

[1] The adapted group contained 7, the unadapted group, 12 animals.
[2] Standard error of the mean.

sponse is not a necessary factor in cataractogenesis is the fact that weanling animals fed a galactose diet develop complete cataracts in 15 days with an even smaller transient rise in metabolic rate than that seen in xylose-fed animals. The calorigenic response appears to depend upon energy realized from the metabolism of the dietary carbohydrate, and the small magnitude of the calorigenic response during galactose feeding reflects its relatively low rate of utilization.

Dietary intake was significantly greater in the adapted than in the unadapted animals for the first 9 days of feeding. The increased xylose load appears to be one factor in the increased susceptibility of these animals, but it may not be the only one. Adapted animals when fed high-carbohydrate diets have an enhanced rate of lipid accumulation.[4] Experimental conditions which stimulate lipogenesis have also been shown to enhance the activity of the hexose monophosphate shunt pathway of glucose oxidation (Felts et al., '53; Tepperman and Tepperman, '58; Winegrad and Renold, '58). Several investigators (Darby and Day, '39; Patterson, '55; Heggeness, '60) have suggested that xylose may affect the lens in a manner similar to galactose. At least one feature of galactose toxicity is an inhibition of the hexose monophosphate shunt in the lens (Lerman, '59). Recent biochemical studies[5] indicate that xylose may also exert its cataractogenic effect by an inhibition of the shunt but the locus of this inhibition is not at the same enzymatic site as has been shown to occur with galactose. Further studies are indicated to determine whether this type of adaptation evokes stimulation of a carbohydrate oxidative pathway that has significance in xylose cataractogenesis. This method of enhancing the toxicity of xylose may be of value in future studies of lens changes during xylose feeding.

Since the total xylose load remains unchanged and the calorigenic response does not appear to be an intrinsic part of the reversibility of the lens changes, the mechanism responsible for this phenomenon appears to lie in the lens itself. The reported strain differences in susceptibility to xylose cataractogenesis may be due to genetic differences in the significance of specific metabolic pathways in the lens.

SUMMARY

1. Appearance and regression of lenticular changes in xylose-fed weanling rats occurred in the presence of a relatively constant xylose load.

2. The cataractous changes were not dependent on the calorigenic effect of high-carbohydrate intakes in weanling animals. The lenticular changes were more severe in animals adapted to the diet in a manner that abolishes this elevation in oxygen consumption.

3. The mechanism by which regression of the xylose-induced lenticular changes took place appears to reside in the lens itself.

ACKNOWLEDGMENT

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LITERATURE CITED

Darby, W. J., and P. L. Day 1939 Xylose as a cataractogenic agent. Proc. Exp. Biol. Med., *41:* 507.

——— 1940 Blood sugar levels in rats receiving the cataractogenic sugars galactose and xylose. J. Biol. Chem., *133:* 503.

Felts, J. M., R. G. Doell and I. L. Chaikoff 1956 The effect of insulin on the pathways of conversion of glucose to fatty acids in the liver. Ibid., *219:* 473.

Heggeness, F. W. 1960 Response of three strains of albino rats to xylose and galactose. Am. J. Physiol., *198:* 625.

Hiatt, H. H. 1957 Glycogen formation via the pentose phosphate pathway in mice *in vivo*. J. Biol. Chem., *224:* 851.

Lerman, S. 1959 Enzymatic factors in experimental galactose cataract. Science, *130:* 1473.

Miller, M. M., and H. B. Lewis 1932 Pentose metabolism. The rate of absorption of D-xylose and the formation of glycogen in the organism of the white rat after oral administration of D-xylose. J. Biol. Chem., *98:* 133.

Patterson, J. W. 1955 Cataractogenic sugars. Arch. Biochem. Biophys., *58:* 24.

Tepperman, H. M., and J. Tepperman 1958 The hexose monophosphate shunt and adaptive hyperlipogenesis. Diabetes, *7:* 478.

Tracey, M. V. 1950 A colorimetric method for the determination of pentoses in the presence of hexoses and uronic acids. Biochem. J., *47:* 433.

Van Heyningen, R. 1959 Metabolism of xylose by the lens. 2. Rat lens *in vivo* and *in vitro*. Ibid., *73:* 197.

[4] Heggeness, F. W., unpublished data.

[5] Lerman, S., and F. W. Heggeness, unpublished data.

Watts, D. T., and D. R. H. Gowley 1953 A simple apparatus for determining basal metabolism of small animals in the student laboratory. Proc. Soc. Exp. Biol. Med., *84*: 585.

Winegrad, A. I., and A. E. Renold 1958 Studies on rat adipose tissue *in vitro*. II. Effects of insulin on the metabolism of specificially labeled glucose. J. Biol. Chem., 233: 273.

Metabolic Patterns in Preadolescent Children

III. SULFUR BALANCE ON THREE LEVELS OF NITROGEN INTAKE[1]

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Studies concerning sulfur metabolism in preadolescent children are few. Beach et al. ('42) have presented data on 7 boys and one girl, 8 to 12 years old. Others[2] (Folin, '06; Swendseid, '56) have examined sulfur excretion and retention in adults.

Lowe and Pessin ('57) have suggested the desirability of including sulfur determination along with nitrogen balance as a measuring technique for protein anabolism in children. In the present study conducted with preadolescent girls during the years 1954 and 1958, data on sulfur intake, excretion and retention on varying levels of nitrogen intake have been obtained.[3]

EXPERIMENTAL

Five girls were studied in the fall of 1954 at Louisiana State University. Five subjects were studied in the summer of 1958 at Virginia Polytechnic Institute. All subjects were apparently in a normal state of health and development for age, as shown by previous dietary records and a pediatrician's examination.

In 1954, the subjects were fed a diet which provided, daily, 2,000 Cal., 9.60 gm of nitrogen and other nutrients to meet National Research Council recommended allowances (Food and Nutrition Board, '58) for girls 7 to 9 years old. The diet fed in 1958 provided 2,100 Cal., and two levels of nitrogen intake, analyzed as averages of 3.58 and 2.99 gm, respectively. Other nutrients met National Research Council recommendations.

The 1954 study covered a total of 16 consecutive 4-day periods, but analyses are reported here for periods two, 4 and 10 only; these three periods constituted level 3. In 1958, experimental collections of food, feces and urine were made during 8 consecutive 6-day periods, analyses being reported for the 5 subjects on the entire 48 days. The last three periods of the 1958 study were averaged for level 1, the first 5 for level 2. Detailed descriptions of the studies have been published in Southern Cooperative Series Bulletin no. 64 ('59).

Sulfur analysis was done on composite samples previously air-dried at 50°C and burned in an Emerson bomb calorimeter, using a modification of the method given in Piper ('50).

RESULTS

Average daily sulfur values obtained for each level of nitrogen ingested are presented in table 1.

With nitrogen intakes averaging 2.89, 3.52 and 11.15 gm daily, sulfur intakes averaged 0.2313, 0.2674, and 0.7610 gm. The ratio of N:S consumed remained relatively constant with increasing protein intake, averaging 12.5, 13.2 and 14.6. Urinary output of sulfur on the same levels of nitrogen intake averaged 0.1257, 0.1532 and 0.3294 gm, respectively. Fecal ex-

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

[2] McTeer, M. 1957 Studies on protein metabolism with emphasis on sulfur balance. Doctor of Philosphy Dissertation. University of Wisconsin, Madison.

[3] Some of the data presented herein are taken from theses submitted by Joyce T. O'Brien and Polly G. Martin to the Graduate School of Louisiana State University for the degree of Master of Science.

TABLE 1

Average daily balance values of nitrogen and sulfur for 5 preadolescent children on each of three levels of nitrogen intake

Level	Nitrogen	Sulfur	N:S ratio	Nitrogen	Sulfur	N:S ratio
	gm	*gm*		*gm*	*gm*	
	Intake			Urinary excretion		
1	2.89	0.2313	12.5	1.74	0.1257	13.8
2	3.52	0.2674	13.2	2.07	0.1532	13.5
3	11.15	0.7610	14.6	8.38	0.3294	25.4
	Fecal excretion			Retention		
1	0.73	0.0724	10.1	0.42	0.0332	12.6
2	0.73	0.0713	10.2	0.58	0.0422	13.7
3	1.00	0.0787	12.7	1.80	0.3530	5.1

TABLE 2

Statistical analysis of sulfur data of 5 preadolescent children on each of three levels of nitrogen intake

Analysis of variance

Sources of variation	F values		
	Excretion		Retention
	Urinary	Fecal	
Nitrogen levels	73.5[1]	2.2	96.4[1]
Periods within levels	3.7[1]	2.3[2]	9.3[1]
Subjects within periods	2.8[1]	1.4	2.0

Correlation coefficients for sulfur with nitrogen

	Excretion		Retention
	Urinary	Fecal	
Levels 1 and 2	0.88[1]	0.77[1]	0.78[1]
Level 3	0.23	0.41	0.04

[1] Significant at 1% level of probability.
[2] Significant at 5% level of probability.

cretion of sulfur was 0.0724, 0.0713 and 0.0787 gm in the same order, and the amount of sulfur retained averaged, 0.0332, 0.0422 and 0.3530 gm for the three levels of nitrogen intake.

Although the total amounts of sulfur in the feces remained relatively constant with increasing nitrogen intakes, the urinary excretion of this constituent increased with increasing intake. Both sulfur and nitrogen showed higher retention with increased intake, but not in the same proportion; in relation to intake, more sulfur than nitrogen was retained (N:S retention ratio, table 1).

Statistical analysis of variance of the data (table 2) indicated highly significant differences in urinary sulfur excretion and retention on the three levels of nitrogen intake, but not in fecal sulfur excretion. Statistical correlations calculated between nitrogen and sulfur with regard to urinary excretion, fecal excretion and retention indicated a significant correlation only at the low levels of nitrogen intake.

DISCUSSION

The unexpectedly high retention of sulfur as compared with nitrogen (ratio, 5.1) on high nitrogen intake might be interpreted to imply that tissue saturation with protein preceded tissue saturation with sulfur. Macy ('42) noted a N:S retention ratio of 9.1 for children fed an even higher nitrogen intake of 13.17 gm daily; she suggested it might be in part a result of undetermined cutaneous sulfur loss.

In suggesting the measurement of sulfur along with nitrogen balance, Lowe and

TABLE 3

Relationship between observed and theoretical nitrogen balances for 5 preadolescent children on each of three levels of nitrogen intake

Level	Observed sulfur retention	Observed nitrogen balance	Theoretical nitrogen balance[1]
	mg/day	*gm/day*	*gm/day*
1	0.0332	0.42	0.48
2	0.0422	0.58	0.61
3	0.3530	1.80	5.12

[1] Calculations of theoretical nitrogen balance made from observed sulfur retention by the following conversion (Lowe and Pessin, '57): S gm × 14.5.

Pessin ('57) stated that theoretical nitrogen balance calculated from sulfur retained should show a reasonable quantitative relationship to the observed nitrogen balance. Such a test of the present data is shown in table 3. In levels 1 and 2, the theoretical nitrogen balances showed a close relationship to the observed nitrogen balance; but in level 3, the high sulfur retention was clearly not reflected in the quantity of nitrogen retained.

SUMMARY

Sulfur balance studies were carried out with 10 girls, 7 to 9 years old, for varying lengths of time, and with daily nitrogen intakes of 2.89, 3.52 and 11.15 gm. Average N:S intake ratios were 12.5, 13.2 and 14.6. Average urinary N:S ratios were 13.8, 13.5 and 25.4; average fecal N:S ratios were 10.1, 10.2 and 12.7, and for retention, 12.6, 13.7 and 5.1 for the three levels in ascending order. Approximately one eighth of the total sulfur intake was retained on low nitrogen intake levels, and about one half of the total sulfur intake was retained when nitrogen intake was as high as 11 gm daily. Highly significant differences existed between levels of nitrogen fed in both urinary excretion of sulfur and sulfur retained, but not in fecal sulfur excretion. A highly significant correlation existed between sulfur and nitrogen excreted in both the urine and the feces, and between the sulfur and nitrogen retained when the amount of nitrogen ingested was low; when nitrogen intake was high, correlations were not significant.

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LITERATURE CITED

Beach, E., D. M. Teague, O. Hoffman, B. Munks, F. Hummel, H. Williams and I. Macy 1942 The sulfur metabolism of children. J. Nutrition, *24:* 257.

Folin, O. 1906 Approximately complete analysis of thirty normal urines. Am. J. Physiol., *13:* 45.

Food and Nutrition Board 1958 Recommended Dietary Allowances. Publication 589, National Academy of Sciences—National Research Council, Washington, D. C.

Lowe, C. U., and M. A. Pessin 1957 The conduct of clinical trials of substances proposed for the nutrition of infants and children. Pediatrics, *19:* 694.

Macy, I. G. 1942 Nutrition and Chemical Growth in Childhood, vol. 1. Evaluation. Charles C Thomas, Springfield, Illinois.

Piper, C. S. 1950 Soil and Plant Analysis. Interscience Publishers, Inc., New York.

Swendseid, M. E., I. Williams and M. S. Dunn 1956 Amino acid requirements of young women based on nitrogen balance data. I. The sulfur containing amino acids. J. Nutrition, *58:* 495.

Southern Cooperative Series, Bull. no. 64. 1959 Metabolic patterns in preadolescent children. I. Description of metabolic studies. Technical Committee, Southern Regional Nutrition Research Project (S-28).

The Effect of Animal Protein and Vegetable Protein Diets Having the Same Fat Content on the Serum Lipid Levels of Young Women[1]

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In nutritional studies of vegetarians, Hardinge and Stare ('54) found lower serum cholesterol levels among those who were pure vegetarians than among lacto-ovovegetarians. Both groups had significantly lower levels than nonvegetarians. Olson et al. ('58), by reducing the protein content of the diet from 100 gm (17% of total calories), mainly from animal sources, to 25 gm (4% of total calories) of vegetable proteins, were able to decrease the serum cholesterol level significantly in human subjects. The fat content of the diet, 80 gm of butterfat or 30% of total calories, remained the same. Keys and Anderson ('57) found no significant change in serum cholesterol levels when a group of healthy men were changed from a diet containing about 83 gm or 8.6% of the total calories as protein to one containing about 130 gm or 17.7%. The fat intake was kept constant at 20% of total calories. Albanese et al. ('59) fed a high-protein supplement containing milk proteins to older convalescent patients which changed their protein intake from 14% of their total caloric intake to 19%. This frequently increased the serum cholesterol levels. Fat intake of the patients was about 25% of their total caloric intake. The authors suggest that protein may have lipotropic activity during the growth period, but that in mature animals this activity is reduced and, in some cases, the protein supplement may induce cholesterolgenic effects.

Leveille and Fisher ('58) found a higher level of serum cholesterol in growing chickens fed a diet containing 8% protein than in those receiving a 25% protein diet, although the chickens having the lower protein intake received only 0.3% of cholesterol in the diet, whereas the higher level protein diet contained 2% of cholesterol. Kokatnur et al. ('58) using male, mature chickens noted that a high-fat diet increased serum cholesterol values, but only when the level of protein in the diet was inadequate. Jones and Huffman ('56) observed that either reduction or elevation of casein in their diets produced hypercholesterolemia in rats. The optimum level of casein was 12 to 18% of the diet. They suggest that different mechanisms operate in the two cases.

In an experiment dealing with the relationship of vitamin B_{12} to protein utilization, a diet of 40 to 45 gm of protein per day, about 85% vegetarian in origin, was fed for 6 weeks to 20 young women subjects.[4] Serum cholesterol levels were determined during the week before the start of the diet and at two-week intervals thereafter. After the subjects had eaten the diet for two weeks, a drop in serum cholesterol levels from 190 ± 3 to 174 ± 5 mg/100 ml occurred. From then to the end of the 6th week the decline to a level of 166 ± 5 was slight but still significant. The drop in levels for the whole 6-week period was

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[1] This study was part of a Northeast Regional Project NE-37, Relationships between Protein and Other Selected Nutrients and their Metabolism and Utilization; a cooperative study involving agricultural experiment stations in the Northeast Region, and supported in part by regional funds of the United States Department of Agriculture.

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highly significant ($P < 0.001$). About 8% of the total calories was from protein and about 28% was provided by fat of mixed origin. Roughly, 12% of the fat calories was from animal sources, 12% from salad oil and the rest was from margarine, hydrogenated shortening and traces of fat in the foods. The question was raised as to whether the amount and origin of the protein or the low level of fat produced the lower cholesterol levels. The present experiment was designed to test, using human subjects, the difference in effect on serum lipids of two protein diets, one deriving its protein content from vegetable sources and one from animal sources. The mixed fat content of the two diets was the same in amount and origin.

EXPERIMENTAL

Twelve young women students were selected from a group of volunteers following a physical examination by the University physician. Their average age was 18.6 with a range from 17 to 22 years, and their average weight was 58 kg with a range from 49 to 66 kg.

The experiment was conducted for 6 weeks. Six subjects were assigned at random to each diet. All portions of food served were weighed.

Menus for one week were developed for each diet, and these menus were repeated throughout the 6-week period. Typical daily menus are shown in table 1. The protein content of both diets averaged 45 to

TABLE 1

Typical daily menus

Vegetable-protein diet		Animal-protein diet	
	gm		*gm*
		Breakfast	
Citrus juice	100	Citrus juice	100
Whole milk	120	Whole milk	120
Wholegrain cereal	28	Skim milk	120
Flour biscuits[1]	84	Cornstarch wafers[2]	36
Margarine	7	Margarine	7
Coffee cream	30	Coffee cream	30
Sugar	4	Sugar	4
		Lunch	
Rice and mushroom sauce	200	Skim milk	200
Biscuits	84	Lettuce	25
Lettuce	25	Cottage cheese	50
Salad oil (corn)	14	Canned fruit	50
Margarine	7	Salad oil (corn)	14
Canned fruit	100	Margarine	7
		Corn starch wafers	36
		Jelly	40
		Junket or jello	125
		Dinner	
Fruit juice	100	Skim milk	240
Macaroni and vegetable soup	150	Fruit juice	100
Peas or lima beans	75	Veal, turkey or fish	50
Carrot and raisins	40	Lettuce	25
Salad oil (corn)	14	Carrot and raisins	40
Biscuits	56	Salad oil (corn)	14
Margarine	7	Cornstarch wafers	71
Flour wafers[3]	42	Jelly	40
Canned fruit	100	Canned fruit	100
		Margarine	7

[1] Recipe for 24 biscuits: (in grams) flour, 414; soy powder, 81.6; baking powder, 21.0; salt 10.8; Crisco, 87.0; and water 216 to 240 ml.

[2] Recipe for about 90 cornstarch wafers: (in grams) Crisco, 144.6; sucrose, 84.6; Dextri-Maltose, 35.4; cornstarch, 300; salt, 7.2; non-fat dry milk, 28.8; and water plus flavoring, 48 to 72 ml.

[3] Recipe for about 90 flour wafers: (in grams) flour, 300; Crisco, 144.6; sucrose, 84.6; Dextri-Maltose, 35.4; soy powder, 28.8; salt, 7.2; and water plus flavoring, 48 to 72 ml.

50 gm per day or about 8% of the calories. The sources of vegetable protein were rice, macaroni, wheat and oat cereals, legumes and a high-gluten white flour[5] which, with soybean powder,[6] was used to make biscuits. The sources of animal protein were uncreamed cottage cheese, skim milk, baked ground veal patties, steamed turkey breast and steamed cod fillets. Gelatin and skim milk desserts were also used. As no flour could be used in the animal protein diet because of the vegetable protein it contained, a wafer of cornstarch, dry skim milk, dextri-maltose,[7] hydrogenated vegetable shortening[8] and flavoring was prepared.

The fat content of both diets was approximately 36% of the calories or an average of 95 gm. It consisted of a margarine[9] made of hydrogenated soybean and cottonseed oils in which the fatty acids were 80% unsaturated and 14.4% polyunsaturated and a hydrogenated vegetable shortening[10] in which the fatty acids were 75% unsaturated and 7.8% polyunsaturated. All subjects had 30 gm of coffee cream containing 5.7 gm of fat, 120 gm of whole milk containing 4.3 gm of fat and 28 gm of salad oil[11] daily. Although as much fat as possible was removed, a small amount averaging 4.3 gm of animal fat or 1.5% of the total calories was present in the animal protein diet daily.

The same low-protein vegetables, fruits and juices were used for both diets. To avoid variability all of the food, except a few fresh fruits and vegetables, was bought in quantities to supply the experiment.

Subjects were weighed daily and adjustments in calories were made to prevent weight changes. This was done by adding or subtracting jelly or sugar and margarine to keep the percentage of calories from fat constant per individual. The average intake for the group was 2370 Cal.

Vitamin and mineral supplements were given to meet the daily allowances recommended by the National Research Council (Food and Nutrition Board, '58). Dicalcium phosphate tablets,[12] each containing 120 mg of calcium were given to all subjects, the vegetable-protein group receiving 4 tablets daily and the animal-protein group, two tablets daily. Capsules[13] containing minerals and vitamins were used, one daily for each subject.

A composite representing the total daily food intake of one subject was saved for each of the 14 different menus. This was homogenized in a Waring blender, brought to a known volume and an aliquot taken for total nitrogen determination. The macroKjeldahl procedure was used. Nitrogen determinations were also made on individual samples of the high-protein foods, especially dairy foods, as no comparable figures could be obtained from food tables. Fat as total ether-soluble extracts was determined by Sohxlet extraction (Joslyn, '50). The fat content of individual foods, especially the meats, milk, cream, cottage cheese, wafers and biscuits was determined.

The energy value of the diets was calculated using U.S.D.A. Agriculture Handbook no. 8 (Watt and Merrill, '50) with the exception of that of the original recipes prepared for biscuits and wafers. For these the energy factors were used in accordance with the method for calculating energy described in Handbook no. 8.

Venous blood samples were taken immediately before lunch at the start of the experiment and at the end of every week thereafter. The samples were centrifuged and the serum was removed and frozen. Serum cholesterol, serum lipid phosphorus and serum fatty esters were determined using methods developed by Clayton et al. ('59). Tripalmitin was used as the standard in the serum fatty ester determinations, and the results are therefore expressed as mg/100 mg of tripalmitin.

RESULTS AND DISCUSSION

Table 2 records the effects on the serum cholesterol of the subjects consuming the

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[5] Pillsbury's Balancer High Gluten Flour.

[6] So Bee Hypoallergenic Formula Powder, Instant.

[7] Dextri-Maltose, Mead-Johnson and Company, Evansville, Indiana.

[8] Crisco.

[9] Blue Bonnet.

[10] Crisco.

[11] Mazola, Corn Products Refining Company, New York.

[12] VitaKaps-M and di-calcium phosphate tablets were a gift from Abbott Laboratories, North Chicago, Illinois.

[13] See footnote 12.

TABLE 2

Average serum lipid levels during ingestion of animal protein and vegetable protein diets having the same fat content (six subjects assigned to each diet)

Weeks	Animal-protein diet Cholesterol	Vegetable-protein diet Cholesterol
	mg/100 ml	*mg/100 ml*
0	188.8 ± 11.36[1]	182.1 ± 17.56
2	168.1 ± 7.73	140.5 ± 8.36
3	160.8 ± 10.83	138.9 ± 7.82
4	153.8 ± 6.77	140.3 ± 6.68
5	160.9 ± 7.44	141.6 ± 7.62
6	157.1 ± 8.84	137.3 ± 7.98

[1] Standard error.

vegetable or animal protein diets. At the end of two weeks on the two diets, covariance analysis showed that the serum cholesterol levels of the subjects receiving the vegetable protein diet were significantly lower than those eating the animal protein diet ($P < 0.005$). The difference was not significant at the third, 4th and 6th weeks, but was at the 5th week ($P < 0.025$). The levels of three girls in each group increased in the 5th week, more for those in the animal-protein group than for those in the vegetable-protein group. This happened to be the week of midterm examinations. The two girls who showed the highest levels (241 and 263 mg/100 ml) at the beginning of the study had levels at the end which had almost reached those of the other girls (165 and 183 mg/100 ml). One was in the animal-protein group and the other in the vegetable group. The levels of the group eating the vegetable-protein diet remained lower than those receiving the animal-protein diet from the end of the first two weeks until the end of the experiment.

Calculations using the tables in The Amino Acid Content of Foods (Orr and Watt, '57) showed that the animal-protein diet contained more methionine (1.2 gm per day) than the vegetable-protein diet (0.5 gm per day).

It may be that differences in the availability of the amino acids in the two diets affected the actual quantities of the amino acids utilized, although the weight in grams of protein was the same for both. The availability of 8 essential amino acids and cystine and tyrosine in a low-protein diet and in diets containing either egg, pork or peanut butter as the chief source of nitrogen was determined by Watts et al. ('59) using young men as subjects. With the exception of tyrosine, they reported that the average values for the availability of the amino acids in the peanut butter diet were lower than those for the pork or the egg diets.

Covariance analysis showed that the serum lipid phosphorus levels of the vegetable-protein group were significantly lower than those of the animal-protein group at the end of two weeks ($P < 0.05$). There was no difference during the remaining 4 weeks of the experiment. There was no significant difference between the serum fatty ester levels of the two groups during the 6 weeks.

A "t" test, using individual differences, indicated a significant drop in serum cholesterol levels in both groups during the 6-week diet period ($P < 0.02$). There was also a drop in the serum lipid phosphorus level of the vegetable-protein group ($P < 0.05$), but the level of the animal-protein group did not fall significantly. There was no appreciable change in the serum fatty ester level of either group during the experiment.

The overall reduction in serum cholesterol produced by both diets may be due, in part, to the fact that a large percentage of the fat in the diets was of vegetable origin in the form of hydrogenated vegetable shortening, margarine and corn oil. The corn oil, which supplied 30% of the fat, contains unsaturated fatty acids, essential fatty acids and sitosterol. All of these have been suggested as factors involved in the reduction of serum cholesterol levels. Ahrens ('57) has stated that the number of double bonds in dietary fats is the main factor in lowering serum cholesterol levels. Kinsell and Sinclair ('57), however, have suggested that the primary determining factor is the presence of essential fatty acids. Beveridge et al.[14] advanced the theory that the sterol content of corn oil is responsible for its depressant effect on serum cholesterol levels.

[14] Beveridge, J. M. R., W. F. Connell and G. A. Mayer 1957 Cholesterol depressant factor in corn oil. Federation Proc., *16:* 11 (abstract).

SUMMARY

Serum cholesterol, serum lipid phosphorus and serum fatty esters were determined for 12 young women, 6 of whom consumed a diet containing 50 gm of protein from vegetable sources, while the diet of the other 6 subjects contained 50 gm of protein from animal sources. The mixed fat content of the two diets was the same in quantity (95 gm) and origin.

At the end of two weeks and 5 weeks on the dietary regimen, the serum cholesterol levels of the subjects receiving the vegetable-protein diet were significantly lower than those eating the animal-protein diet. They were also lower at the third, 4th and 6th weeks, but the differences were not significant.

Serum lipid phosphorus levels of the vegetable-protein group were lower than those of the animal-protein group at the end of two weeks, but there was no difference during the remaining 4 weeks.

There was no difference between the serum fatty ester levels of the two groups during the 6 weeks.

There was a significant decrease in the serum cholesterol levels of both groups during the 6 weeks. Also a small decrease was observed in the serum lipid phosphorus levels of the vegetable-protein group, but no change in the serum lipid phosphorus levels of the animal-protein group nor in the serum fatty ester levels of either group during the study.

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LITERATURE CITED

Ahrens, E. H., Jr. 1957 Nutritional factors and serum lipid levels. Am. J. Med., *23:* 928.

Albanese, A. A., R. A. Higgons, E. J. Lorenze and L. A. Orto 1959 Effect of dietary protein on blood cholesterol levels of adults. Geriatrics, *14:* 237.

Clayton, M. M., P. A. Adams, G. B. Mahoney, S. W. Randall and E. T. Schwartz 1959 Micro methods for the determination of chylomicron counts, fatty esters, lipid phosphorus and cholesterol in blood serum. Clin. Chem., *5:* 426.

Food and Nutrition Board 1958 Recommended dietary allowances, pub. 589. National Acad. of Sciences—National Research Council, Washington, D.C.

Hardinge, M. G., and F. J. Stare 1954 Nutritional studies of vegetarians. 2. Dietary and serum levels of cholesterol. J. Clin. Nutrition, *2:* 83.

Jones, R. J., and S. Huffman 1956 Chronic effect of dietary protein on hypercholesteremia in the rat. Proc. Soc. Exp. Biol. Med., *93:* 519.

Joslyn, M. A. 1950 Methods in Food Analysis Applied to Plant Products. Academic Press, Inc., New York, ch. 6.

Keys, A., and J. T. Anderson 1957 Dietary protein and serum cholesterol level in man. Am. J. Clin. Nutrition, *5:* 29.

Kinsell, L. W., and H. M. Sinclair 1957 Fats and disease. Lancet, *1:* 883.

Kokatnur, M., N. T. Rand, F. A. Kummerow and H. M. Scott 1958 Effect of dietary protein and fat on changes of serum cholesterol in mature birds. J. Nutrition, *64:* 177.

Leveille, G. A., and H. Fisher 1958 Plasma cholesterol in growing chicken as influenced by dietary protein and fat. Proc. Soc. Exp. Biol. Med., *98:* 630.

Olson, R. E., J. W. Vester, D. Gursey, N. Davis and D. Longman 1958 The effect of low-protein diets upon serum cholesterol in man. Am. J. Clin. Nutrition, *6:* 310.

Orr, M. L., and B. K. Watt 1957 Amino acid content of foods. Home Economics Research Report no. 4. Agricultural Research Service, Washington, D.C.

Watt, B. K., and A. L. Merrill 1950 Composition of Foods—Raw, Processed, Prepared. Agricultural Handbook no. 8. U.S.D.A., Washington D.C.

Watts, J. H., L. K. Booker, J. W. McAfee, E. G. Williams, W. G. Wright and F. Jones, Jr. 1959 Biological availability of essential amino acids to human subjects. I. Whole egg, pork muscle and peanut butter. J. Nutrition, *67:* 483.

The Antisterility Activity of Alpha-Tocohydroquinone in the Female Rat[1]

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In 1950, Mackenzie and associates reported the prevention and cure of muscular dystrophy in the rabbit with α-tocohydroquinone. The activity of the hydroquinone in curative tests differed in two important respects from the activity of α-tocopherol (Mackenzie and McCollum, '40; Mackenzie, '42). First, the hydroquinone was more active when injected intravenously than when given orally. Second, the duration of the effect of the injected hydroquinone was not proportional to the dose. Nevertheless, 5 mg of the hydroquinone administered intravenously possessed the same antidystrophy activity as α-tocopherol itself.

These observations reopened the question of the antisterility activity of α-tocohydroquinone in the pregnant female rat. In 1940, Golumbic and Mattill had reported that the compound was devoid of antisterility activity. Issidorides and Mattill ('51) then injected pregnant rats intravenously with single 25 mg doses of the hydroquinone. The results were again negative. However, in view of the antidystrophy tests, it seemed to us that the hydroquinone might be effective when administered intravenously throughout pregnancy. For this purpose, we employed the disuccinate of α-tocohydroquinone because of its superior stability in air. Although the ester exhibited one-quarter to one-half of the activity of the free hydroquinone in the dystrophic rabbit, it failed to prevent resorption in vitamin E-deficient female rats even when injected at a level of 8 mg daily throughout pregnancy (Mackenzie and Mackenzie, '53).

It appeared that these experiments had disposed of the antisterility activity of α-tocohydroquinone in the pregnant rat. However, in a recent study of muscular dystrophy in the rat, intravenous α-tocohydroquinone disuccinate was found to possess little activity whereas the free hydroquinone was approximately as active as α-tocopherol (Mackenzie and Mackenzie, '59). These results suggested that our failure to observe antisterility activity with the disuccinate might have been due to its slow rate of hydrolysis in the rat. Accordingly, the antisterility activity of the hydroquinone was re-examined by injecting it daily throughout pregnancy.

MATERIALS AND METHODS

Female rats of the Sprague-Dawley strain were supplied with a vitamin E-deficient diet (Mackenzie and Mackenzie, '59) when they had reached a weight of 50 gm. The diet fed one group of rats contained lard which had been rendered from leaf lard at 80° in the laboratory. The diet fed a second group of animals contained stripped lard.[2] When initial fertility was encountered in some of the animals in both groups, all of the rats were carried through a second pregnancy. Resorption gestations resulted in all instances as indicated by the placental sign, a weight gain and a subsequent weight loss.

α-Tocohydroquinone was prepared by the hydrogenation of α-tocoquinone[3] under pressure in the presence of 5% palladium on $CaCO_3$. The hydroquinone was dissolved in propylene glycol-10% ethanol and at the end of the reaction the catalyst was removed by centrifugation. A portion of the supernatant solution was diluted with the same solvent mixture to give a

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[2] Obtained from Distillation Products Industries, Rochester, New York.
[3] The α-tocoquinone was kindly supplied by Dr. Phillip L. Harris of Distillation Products Industries, Rochester, New York.

final concentration of 1.0 mg of α-tocohydroquinone in 0.1 ml, and aliquots of this solution were injected immediately into test animals. Another portion of the supernatant solution was diluted with absolute ethanol and at once analyzed for reducing materials by the method of Emmerie and Engel ('38). A detailed description of the synthetic and analytical procedures has been presented earlier (Mackenzie and Mackenzie, '59). In the present experiment, fresh α-tocohydroquinone was prepared daily in yields of 90% or better.

Daily injections were started on the day sperm were found in the vaginal smears and were continued until the animals were autopsied on day 21 or 22 of pregnancy. Animals were prepared for repeated injections in the tail vein as described in a previous publication (Mackenzie and Mackenzie, '53).

Blocks of striated muscle were fixed in Zenker-formol and stained with hematoxylin and eosin for microscopic examination.

RESULTS AND DISCUSSION

As shown in table 1, the parenteral administration of 2 to 2.5 mg of α-tocohydroquinone failed to prevent fetal resorption. When the daily dose was increased to 5 mg, a positive response was obtained in 4 of 5 animals. Living fetuses were also present at 21 days in a 5th animal injected with 10 mg of the hydroquinone.

Because of the large dose of α-tocohydroquinone required for fertility, the purity of the preparation was a matter of considerable importance particularly since the hydroquinone was prepared from α-tocoquinone which had been prepared in turn from α-tocopherol. Analysis of the α-tocoquinone by the Emmerie and Engel ('38) procedure indicated the presence of approximately 0.6% of reducing material as calculated from an α-tocopherol standard curve. However, this material deviated from the Beer's law relationship at an optical density of 0.3, whereas our α-tocopherol standard still conformed with Beer's law at an optical density of 1.0. Furthermore, infrared analysis of the α-tocoquinone showed no trace of aromatic hydroxyl groups.[4] Tishler and Wendler ('41) have shown that when α-tocohydroquinone is heated for several hours at 100° in the presence of acid, it undergoes ring closure to form α-tocopherol. It is unlikely that such a reaction occurred during the hydrogenation of α-tocoquinone at 26° in the presence of $CaCO_3$.

In view of these considerations, it appears likely that the positive response obtained in the rat fertility test (table 1) was due to α-tocohydroquinone and not to a contaminant. Presumably, Issidorides and Mattill ('51) obtained negative results with α-tocohydroquinone because it was injected only on the first day of pregnancy. Our failure to obtain fertility with massive daily doses of the disuccinate (Mackenzie and Mackenzie, '53) was probably due to its slow rate of hydrolysis in the rat.

[4] We are indebted to Mr. Blum of Distillation Products Industries for the infrared analysis.

TABLE 1

Antisterility activity of parenteral α-tocohydroquinone in the female rat

α-Tocohydroquinone[1]	Route of injection[2]	No. of living young	No. of resorption sites	Maternal muscle lesions
mg/day				
2	IP	0	5	±
2	IP	0	9	±
2.5	IV	0	4	±
2.5	IV	0	3	±
5	IP	9	0	±
5	IP	7	0	—
5	IV	0	1	—
5	IV	1	4	—
5	IV	3	0	—
10	IP	4	0	—

[1] Six control animals received no α-tocohydroquinone. None of them had living young and all had resorption sites. All of the controls had + to ++ muscle lesions.

[2] IP indicaes intraperitoneal; IV, intravenous.

Mason and Rao have also found that α-tocohydroquinone possesses antisterility activity in the female rat.[5] When administered orally during the 5th to 8th days of pregnancy, the hydroquinone exhibited approximately one-twentieth the activity of α-tocopherol.

In the present experiment, doses of α-tocohydroquinone that failed to prevent resorption alleviated the muscle damage associated with vitamin E deficiency (table 1). This antidystrophy activty was in agreement with our observation that the intravenous injection of 1.8 mg of α-tocohydroquinone for three days causes a prompt fall in creatine excretion in the dystrophic rat (Mackenzie and Mackenzie, '59).

The problem of the biochemical activity of α-tocohydroquinone is still unanswered. It is possible that, at the molecular level, α-tocopherol and α-tocohydroquinone have different functions since similarities in their effects on the intact animal may depend on their interconversion *in vivo*. Whereas a clear-cut negative response in the whole animal excludes both a direct effect and conversion to an active form, a positive response does not distinguish between these two possibilities. On the other hand, there is no convincing evidence yet that α-tocopherol is converted to its hydroquinone in biological systems.

SUMMARY

The antisterility activity of α-tocohydroquinone in vitamin E-deficient female rats has been re-examined.

For this purpose, the hydroquinone was prepared daily by the catalytic hydrogenation of α-tocoquinone which was free of α-tocopherol.

When injected at a level of 5 mg daily, the α-tocohydroquinone exhibited antisterility activity. Smaller doses cured the muscle lesions in the mother but did not prevent fetal death and resorption.

ACKNOWLEDGMENT

We wish to express our thanks to Mrs. Myrtle Dudler for her technical assistance.

[5] Personal communication, 1960, Mason and Rao.

LITERATURE CITED

Emmerie, A., and C. Engel 1938 Colorimetric determination of α-tocopherol (vitamin E). Rec. Trav. Chim., *57:* 1351.

Golumbic, C., and H. A. Mattill 1940 The oxidation of vitamin E. J. Biol. Chem., *134:* 535.

Issidorides, A., and H. A. Mattill 1951 The biological activity of α-tocopherylhydroquinone in rats. Ibid., *188:* 313.

Mackenzie, C. G. 1942 The development of E deficiency in rabbits and the response to alpha-tocopherol as measured by creatine excretion. Fed. Proc., *1:* 190.

Mackenzie, C. G., and E. V. McCollum 1940 The cure of muscular dystrophy in the rabbit by alpha-tocopherol and its effect on creatine metabolism. J. Nutrition, *19:* 345.

Mackenzie, J. B., and C. G. Mackenzie 1953 Vitamin E activity of alpha-tocopherylhydroquinone and muscular dystrophy. Proc. Soc. Exp. Biol. Med., *48:* 642.

——— 1959 The effect of α-tocopherol, α-tocopherylhydroquinone and their esters on experimental muscular dystrophy in the rat. J. Nutrition, *67:* 223.

Mackenzie, J. B., H. Rosenkrantz, S. Ulick and A. T. Milhorat 1950 The biological activty of α-tocopherylhydroquinone and α-tocopherylquinone. J. Biol. Chem., *183:* 655.

Tishler, M., and N. L. Wendler 1941 Interrelation of α-tocopherol and α-tocopherylquinone. J. Am. Chem. Soc., *63:* 1532.

Effects of Pantothenic Acid, Pyridoxine and Thiamine Deficiencies upon Antibody Formation to Influenza Virus PR-8 in Rats[1]

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The role of the vitamins in antibody production has been the subject of a continuous investigation in this laboratory. These and other studies demonstrating the dependence of the processes of antibody formation upon an adequate consumption of certain vitamins have been summarized in a recent review (Axelrod, '60). Our investigations, to date, have employed the human erythrocyte and alum-precipitated diphtheria toxoid as antigens. In order to obtain further information on the antigenic specificity of these deficiency states, it was deemed advisable to extend these studies by the application of another antigenic stimulus. Influenza virus was chosen since it represents an antigen of clinical interest and its corresponding serum antibody can be readily determined by a specific hemagglutination inhibition procedure. The comparative infrequency with which viruses have been used as antigens in studies of this nature further prompted this choice.

The present paper records the effects of pantothenic acid, pyridoxine and thiamine deficiencies upon antibody formation to influenza virus PR-8 in the rat.

METHODS AND MATERIALS

I. *Virus preparation.* Pooled, allantoic fluid of chick embryos infected with egg-adapted influenza virus PR-8 was the source of viral antigen in these studies. Eleven-day old white Leghorn embryos were innoculated with 0.1 ml of a 10^{-2} dilution of influenza virus PR-8 in allantoic fluid[2] and incubated at 37°C for 48 hours. Dilutions were made in isotonic saline. Live embryos were then refrigerated overnight and the infected allantoic fluid harvested. The pooled fluid was centrifuged to remove tissue particles and red blood cells and stored at −20°C. Detailed procedures are given by Hirst ('42) and Buddingh ('52).

Adjuvant vaccine was prepared by emulsifying 5 ml of the undiluted, infected, allantoic fluid with 4.5 ml of 60 viscosity mineral oil and 0.5 ml of Arlacel A.[3]

II. *Hemagglutination reaction.* Titration of the erythrocyte agglutinating capacity of influenza virus was performed essentially as described by the Committee on Standard Serological Procedures for Influenza Studies ('50). A stock suspension of chicken blood was prepared by adding 10 ml of blood, obtained by cardiac puncture of young adult hens, to 30 ml of Alsever's solution. Erythrocytes from 10 ml of this suspension were collected by centrifugation, washed three times with 30 ml of isotonic sodium chloride and diluted with isotonic sodium chloride to yield suspensions of the required concentration. Since the quality of the cells may vary from time to time, it is important that the same cells be used in both the hemagglutination and the hemagglutination-inhibition tests. Hemagglutination was determined by the pattern method of Salk ('44). One unit of hemagglutinating activity is contained in 0.25 ml of the highest dilution

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[2] Furnished by Dr. Julius Youngner of the Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh.
[3] Non-toxic fraction obtained from Atlas Powder Co., Wilmington, Delaware.

of antigen, i.e., pooled, infected allantoic fluid, which completely agglutinates the standard 0.5% erythrocyte suspension. Hemagglutinating potency of the antigen was redetermined each time it was used in the hemagglutination-inhibition test.

III. *Hemagglutination-inhibition reaction.* Serum was obtained from anesthetized[4] rats bled by cardiac puncture and nonspecific inhibitors of influenza virus inactivated by incubating 0.1 ml of serum with 0.1 ml of buffered trypsin solution at 56°C for 30 minutes (Sampaio and Isaacs, '53). The buffered trypsin solution was prepared by dissolving 40 mg of trypsin (Difco 1:250 powder) in 5 ml of a 0.1 M phosphate buffer of pH 8.0. Serial twofold dilutions of inactivated sera were made in isotonic sodium chloride solution beginning with a 1:20 dilution. Their hemagglutinating-inhibitory potencies were determined by the method proposed by the Committee on Standard Serological Procedures for Influenza Studies ('50) with the exceptions that 0.5 ml of the diluted sera and 0.25 ml of a 1% suspension of chicken erythrocytes were added to each tube. The test antigen, i.e., infected allantoic fluid, was diluted so that 4 hemagglutinating units were contained in the 0.25 ml added to each tube. A standard antiserum of known viral antibody content was secured by pooling sera from rats immunized with the infected allantoic fluid. Titrations of this standard antiserum, which was stored at −20°C, were conducted simultaneously with each titration of serum of unknown viral antibody content.

In the hemagglutination-inhibition test, specific antibody in immune serum combines with a constant amount of virus in each tube and neutralizes its capacity to agglutinate chicken erythrocytes. The hemagglutination-inhibition titre (H.I.) is the highest initial dilution of serum, i.e., the dilution before addition of antigen and erythrocyte suspension which effects complete inhibition of agglutination. It, therefore, varies directly with serum antibody content. Results are reproducible to one twofold dilution of serum. In each experiment, the same stock antigen was utilized for immunization and determination of serum antibody content.

IV. *Animals and diets.* Male, weanling, albino rats of the Holtzman strain were used. The animals were housed individually in wide-meshed screen bottom cages and fed ad libitum unless stated otherwise. The compositions of the diets used to produce deficiencies of pantothenic acid, pyridoxine and thiamine and of the corresponding control diets have been described previously in detail (Pruzansky and Axelrod, '55). Briefly, all animals received a basal, semisynthetic diet composed of sucrose, casein, salts, lipids and the fat-soluble vitamins. In addition, each rat was fed daily a vitamin supplement in the form of a pill. For the control groups, this pill contained adequate amounts of all the B-vitamins known to be required by the rat. For the deficient groups, only the appropriate vitamin was omitted from the pill (Pruzansky and Axelrod, '55).

In every experiment, the requisite dietary regimen was instituted when the animals arrived at the laboratory and was not varied during the course of study. Severity of the deficiences was judged by the extent of growth inhibition rather than any outward symptoms of deficiency.

EXPERIMENTAL AND RESULTS

In preliminary experiments, we investigated the effects of variations in dosages of antigen and in immunization schedule upon the magnitude of antibody response in adult male rats, fed a laboratory chow[5] diet. These studies demonstrated that three intraperitoneal injections of 1 ml of a 10^{-1} dilution of pooled, infected, allantoic fluid at weekly intervals produced a satisfactory antibody response. The animals were bled for serum antibody determinations one week after the final injection. Since combination of the antigen with adjuvant did not potentiate its immunizing capacity, we used the untreated, pooled, infected allantoic fluid exclusively as the antigenic stimulus in these studies. Rat sera contain considerable quantities of nonspecific inhibitors of the influenza virus hemagglutin. Trypsin treatment of serum, however, inactivated these inhibitors without affecting the serum antibodies produced in re-

[4] By Nembutal.

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

sponse to influenza virus. Repeated analyses failed to demonstrate the presence of antibodies to influenza virus, in non-immunized rats.

1. *Pantothenic acid deficiency.* The effect of a pantothenic acid deficiency upon antibody response was investigated in two experiments (exp. 1 and 2) conducted in identical fashion. After the animals had received the experimental diet for 5 weeks they were immunized as described above. Serum antibody determinations were made one week after the final injection of antigen when the animals had been on experiment for 8 weeks.

At this time, the average weight of the control rats in both experiments was 325 gm, and that of the pantothenic acid-deficient groups was 133 gm. Serum antibody levels are presented in figure 1. The pronounced decrease in the circulating antibody content of pantothenic acid-deficient rats was clearly evident. In many deficient animals, the presence of serum antibodies was not detectable.

2. *Pyridoxine deficiency.* This study consisted of three separate and identical experiments (exp. 3, 4 and 5). Immunization was begun after supplying the experimental diet for 4 weeks and consisted of a total of 4 intraperitoneal injections of 1 ml of antigen at weekly intervals. The animals were bled one week after the last injection. At the conclusion of the experiments, the average weight of all control groups was 335 gm and that of the deficient groups was 115 gm. The resulting serum antibody levels shown in figure 2 reflected a marked decrease in the antibody response of pyridoxine-deficient rats.

A group of paired-weighed pyridoxine-supplemented controls was also used in experiment 4. From the beginning of the experiment, each of the animals was permitted to consume only a limited amount of the basal diet in order to maintain its weight equal to that of a paired ad libitum-fed member in the pyridoxine-deficient group. Frequent weighings of both the inanition controls and their deficient partners were necessary. The final average weight of 114 gm attained by the inanition controls agreed closely with that of the pyridoxine-deficient animals (115 gm). It is to be noted that each control animal received the complete vitamin supplement daily, thus insuring a constant adequate intake of the vitamin B complex. The immunization procedure, begun after 4 weeks, was identical with that described

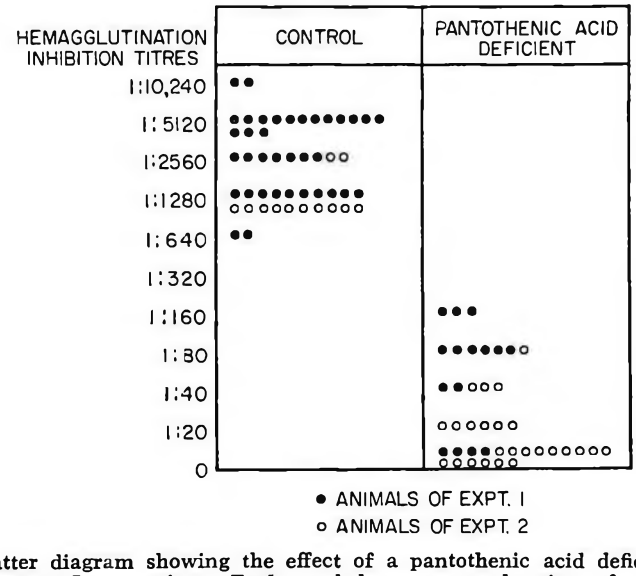

Fig. 1 Scatter diagram showing the effect of a pantothenic acid deficiency upon antibody response to influenza virus. Each symbol represents the titre of an individual rat. In all figures presented in this paper, a hemagglutination-inhibition titre of zero indicates that a 1:20 dilution of serum failed to inhibit the hemagglutinating potency of the antigen.

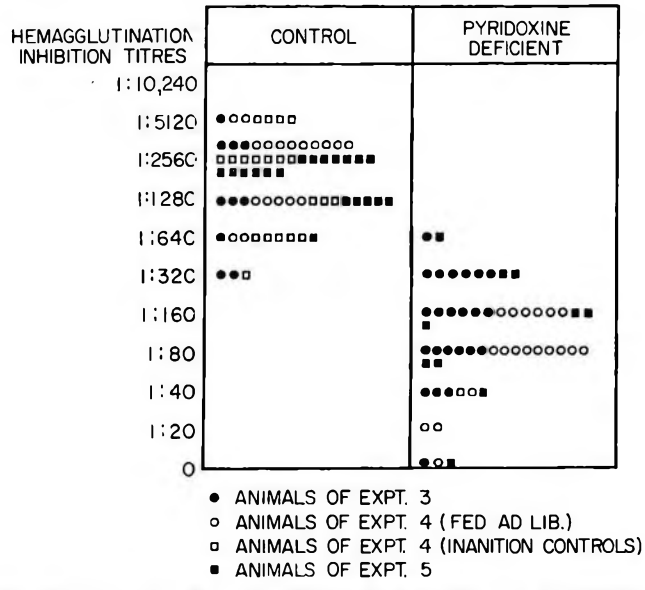

Fig. 2 Scatter diagram showing the effect of a pyridoxine deficiency upon antibody response to influenza virus. Each symbol represents the titre of an individual rat.

in the preceding paragraph. Serum antibody levels obtained one week after the final injection are given in figure 2. Despite the marked weight loss, antibody responses of these animals were comparable to those of the ad libitum-fed control rats

The following experiment was conducted to determine whether the antibody response of pyridoxine-deficient rats could be stimulated by repeated administration of antigen. Nine of the pyridoxine-deficient rats of experiment 4, the antibody responses of which had been determined as described above, continued to receive the deficient diet and were given 4 additional weekly injections of antigen. This supplementary immunization was begun immediately after determination of the original hemagglutination-inhibition titres which ranged from zero to 1:80. Nine of the ad libitum-fed control rats were treated in identical fashion. Antibody determinations were made one week after the final injection of antigen. These 4 additional injections of antigen failed to increase the lowered serum antibody content of the deficient animals and had no further effect upon the already high antibody levels of the control rats.

3. *Thiamine deficiency.* In this experiment (exp. 6), the animals were immunized with only two injections of antigen, the first after 11 days on experiment and the other a week later. Antibody determinations were performed one week after the second injection. This abbreviated immunization was necessitated since it would not have been possible to maintain weanling animals with a thiamine-deficient diet for longer periods. The deficient rats began to lose weight progressively after 11 days on experiment. Many of the animals were in a morbid state when bled for the determination of serum antibody content. At this time, the average weight of the controls was 190 gm and that of the deficient group was 55 gm. As a result of this decreased antigenic stimulus, the antibody response of the controls was lower than that of controls in the previous experiments (fig. 3). However, it is quite apparent that the antibody response of the thiamine-deficient rats was equivalent to that of their corresponding controls (fig. 3).

DISCUSSION

The deleterious effects of specific dietary deficiencies upon antibody formation to a variety of antigenic stimuli have been amply documented (Axelrod and Pruzansky, '55; Axelrod, '60). Other studies em-

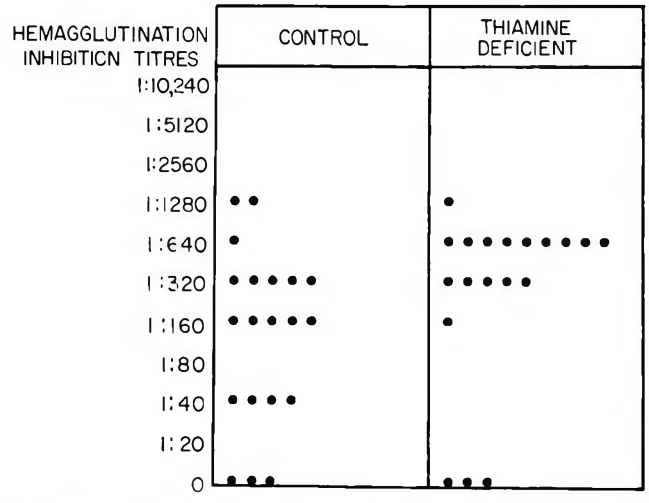

Fig. 3 Scatter diagram showing the effect of a thiamine deficiency upon antibody response to influenza virus. Each symbol represents the titre of an individual rat.

ploying viruses as antigens failed to demonstrate a similar dependence of antibody formation upon adequate consumption of folic acid (Saslaw et al., '46), thiamine and riboflavin (Ruchman, '46) and pyridoxine (Leftwich and Mirick, '49; Mirick and Leftwich, '49). These negative results have been questioned in a critical review (Axelrod and Pruzansky, '55) on the basis of the inconclusive nature of the data presented. A more recent investigation has demonstrated that diets deficient in vitamins A, D and E do not markedly affect production of swine influenza antibodies in mice (Underdahl and Young, '56). The present study presents definite evidence to show that antibody formation to influenza virus PR-8 in the rat is impaired by deficiencies of pantothenic acid and pyridoxine. In this respect, these studies parallel our previous observations made with human erythrocytes and diphtheria toxoid as antigens and further emphasize a more general dependence of antibody production upon adequate nutriture with pantothenic acid and pyridoxine.

It is recognized that the decreased content of circulating antibodies observed in these deficiency states may be due to the presence of abnormal constituents in these sera capable of interfering with the interaction between antibody and antigen in the test system used. This possibility was rendered implausible by recovery experiments showing that the presence of deficient sera did not affect the virus neutralizing capacity of high-titre sera from normal animals.

Results of the current investigation with influenza virus are in agreement with previous findings that thiamine deficiency did not affect antibody response to a variety of antigens. This observation assumes greater significance when it is recalled that the symptoms of thiamine deficiency were far more severe than those produced by the dietary lack of either pantothenic acid or pyridoxine. The failure of a pronounced thiamine deficiency state to affect antibody response argues strongly for the specific nature of the role assumed by certain nutrients, including pantothenic acid and pyridoxine, in the processes of antibody fabrication. This thesis gains further support from the observations made in this and other studies that inanition *per se* is not a detriment to antibody formation.

SUMMARY

The effects of pantothenic acid, pyridoxine and thiamine deficiencies upon antibody production to influenza virus PR-8 have been investigated. Deficient rats were immunized with allantoic fluid infected with influenza virus PR-8 and resulting levels of serum antibody determined with a hemagglutination-inhibition

procedure. Ad libitum-fed and inanition controls were used. High levels of serum antibody were attained in both types of controls. In contrast, antibody production was markedly impaired in pantothenic acid- and pyridoxine-deficient rats. A pronounced thiamine deficiency failed to affect antibody production.

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LITERATURE CITED

Axelrod, A. E. 1960 Nutrition in relation to acquired immunity. Modern Nutrition in Health and Disease, eds., M. G. Wohl and R. S. Goodhart. Lea and Febiger, Philadelphia, p. 550.

Axelrod, A. E., and J. Pruzansky 1955 Role of the vitamins in antibody production. Vitamins and Hormones, *13:* 1.

Buddingh, G. J. 1952 Chick-embryo technics. Viral and Rickettsial Infections of Man, ed., T. M. Rivers. J. B. Lippincott, Philadelphia, p. 109.

Committee on Standard Serological Procedures in Influenza Studies 1950 An agglutination-inhibition test proposed as a standard of reference in influenza diagnostic studies. J. Immunol., 65: 347.

Hirst, G. K. 1942 The quantitative determination of influenza virus and antibodies by means of red cell agglutination. J. Exp. Med., 75: 49.

Leftwich, W. B., and G. S. Mirick 1949 The effect of diet on the susceptibility of the mouse to pneumonia virus of mice (PVM). Ibid., *89:* 155.

Mirick, G. S., and W. B. Leftwich 1949 The effect of diet on the susceptibility of the mouse to pneumonia virus of mice (PVM). Ibid., *89:* 175.

Pruzansky, J., and A. E. Axelrod 1955 Antibody production to diphtheria toxoid in vitamin deficiency states. Proc. Soc. Exp. Biol. Med., *89:* 323.

Ruchman, I. 1946 The effect of nutritional deficiencies on the development of neutralizing antibodies and associated changes in cerebral resistance against the virus of Western Equine Encephalomyelitis. J. Immunol., 53: 51.

Salk, J. E. 1944 A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. Ibid., *49:* 87.

Sampaio, A. A. de C., and A. Isaacs 1953 The action of trypsin on normal serum inhibitors of influenza virus agglutination. Brit. J. Exp. Path., 34: 152.

Saslaw, S., H. E. Wilson, C. A. Doan, O. C. Woolpert and J. L. Schwab 1946 Reaction of monkeys to experimentally induced streptococcus hemolyticus, group C, infection. J. Exp. Med., *84:* 263.

Underdahl, N. R., and G. A. Young 1956 Effect of dietary intake of fat-soluble vitamins on intensity of experimental swine influenza virus infection in mice. Virology, 2: 415.

Observations on Protein Digestion In Vivo

III. RECOVERY OF NITROGEN FROM THE STOMACH AND SMALL INTESTINE AT INTERVALS AFTER FEEDING DIETS CONTAINING DIFFERENT PROTEINS[1]

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Although the overall digestibility and nutritive value of many food and feed proteins have been determined (Mitchell, '48), there have been few direct studies of the rates of digestion of different proteins *in vivo*. The rates of digestion of many proteins have also been studied *in vitro* but such methods do not take into account certain factors that affect protein digestion and absorption in the animal body. The influence of some of the factors that affect the rate of digestion of casein *in vivo* was the subject of a previous report (Peraino et al., '59).

In 1911, London and Rabinowitsch, using dogs with fistulas of the stomach, jejunum or ileum, showed that, of a series of proteins tested, elastin, gelatin and gliadin were the least rapidly digested and that blood and meat proteins were the most rapidly digested in both the stomach and the small intestine. Recently Geiger ('51) and Geiger et al. ('52; '58), using purified diets, studied the rates of digestion of proteins in the rat. They concluded that casein disappeared from the gastrointestinal tract more rapidly than zein, but less rapidly than meat and fish proteins. Gupta et al. ('58) measured the rates of disappearance of a few proteins and an amino acid mixture from the gastrointestinal tract of the rat. They reported that casein, beef proteins, and an amino acid mixture disappeared from the gastrointestinal tract at similar rates, but zein disappeared more slowly. Geiger et al. ('58) and Gupta et al. ('58) recovered a smaller percentage of the ingested nitrogen from the gastrointestinal tract after feeding protein than Dreisbach and Nasset ('54) did in similar experiments. Nasset et al. ('55) concluded from experiments on dogs fed egg albumin, zein or protein-free diets "that the qualitative amino acid composition of intestinal contents is not greatly altered by changing from a non-protein to a protein-containing test meal." Nasset ('57) has ascribed nutritional significance to the observation that the amino acid composition of the intestinal contents is relatively constant, and has suggested that the high rate of turnover of endogenous nitrogen in the small intestine may be sufficient to "obscure any amino acid peculiarity of the food protein." Harper and Kumta ('59) observed a depression in food consumption within 3 to 4 hours after feeding rats a diet in which an amino acid imbalance had been created; so, even though the turnover of endogenous nitrogen in the intestine may be quite large, it does not prevent the signs of an amino acid deficiency from becoming evident in a short time. If, as seems probable, amino acids from the dietary protein are absorbed rapidly, they would not accumulate in the intestine of the rat and would not greatly alter the endogenous amino acid pattern.

The present investigation was undertaken to compare the rates of digestion of several proteins *in vivo* in the rat, and to provide the groundwork for more detailed studies of the reasons for differences in the rates of digestion of different proteins.

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[2] Fellow of the Williams-Waterman Fund Research Corporation, New York.

EXPERIMENTAL

The experimental procedure used was described by Peraino et al. ('59). Male albino rats weighing approximately 200 gm were trained to consume their total daily ration within a single two-hour period. Initially the rats lost weight, but after two weeks on this regimen they had regained their starting weights, were consuming 10 to 14 gm of food per day, and were considered to be suitable for experiment. The training diet contained (in per cent): casein, 15; salt, 5 (Harper, '59); corn oil, 5; water-soluble vitamin mixture, 0.25 (Harper et al., '56); choline chloride, 0.15; and dextrin, 74.6. Fat soluble vitamins A, D and E were included in the corn oil to provide the following concentrations per 100 gm of diet: A, 400 I.U.; D, 40 I.U.; and E, 10 mg.

Most of the experimental diets contained (in percent): dextrin (autoclaved corn starch), 60; protein, 30; corn oil, 5; and salts, 5. A protein content of 30% was not considered excessive yet was thought to be sufficiently high to cause a measurable accumulation of nitrogen in the intestines of rats receiving less digestible proteins. In experiments in which the percentage of protein was altered, dextrin and protein together made up 90% of the diet. Vitamins were not included in the experimental diets because the experiments were completed in 8 hours, and in a previous investigation the omission of vitamins did not affect the results.

The proteins studied were: beef (N = 15.2%) and pork (N = 14.9%) proteins; casein[3] (N = 14.0%); gliadin[4] (N = 15.1%); zein[4] (N = 14.1%); soybean protein[5] (N = 14.1%); gelatin[6] (N = 16.0%); egg albumin[7] (N = 11.9%) and a mixture of L- and DL-amino acids calculated to provide the L-forms in quantities equal to 1.5 times the essential amino acid requirements of the rat, together with nonessential amino acids, primarily glutamic acid, to make a total of 30% of amino acids in the diet (N = 10.6). These proteins and the amino acid mixture were tested at the indicated level without attempting to make them isonitrogenous. The casein was extracted with ethanol. To prepare the beef and pork proteins, the fresh meats were cooked, by either autoclaving (2½ hours, 15 minutes/pound at 15 psi) or roasting (beef, 2½ hours, 7 minutes/pound at 190°C; pork, 4½ hours, 15 minutes/pound at 230°C); dried, and then extracted with petroleum ether to remove fat. The particle size in each case was similar to that of commercial casein.

On the day of the experiment each rat was offered 5 gm of the experimental diet. Well-trained rats that would eat 5 gm of a diet containing 15% of casein in 20 minutes would not eat 5 gm of diets having a higher protein content in this time; therefore, a 30-minute feeding period was used. Even then they would not readily eat 5 gm of diets containing gelatin, zein, egg albumin or free amino acids, but had to be fed nutritionally-adequate diets containing these nitrogen sources for 2 to 4 days before the experiment. A sufficient number for the experiment would then eat 5 gm of all except the amino acid diet in 30 minutes. Only after a 10-day adaptation period were rats able to eat the desired quantity of the amino acid diet in the regular feeding period. Spilled food, collected on paper towels placed under the cages, was returned to the food cups before the time limit had been reached. Rats that did not eat the full quantity in the allotted time were not used.

Rats were killed by chloroform anesthesia at the end of the feeding period (zero time) and at intervals (5 to 10 rats at each interval) thereafter as indicated in the results. The stomach and small intestine of each rat were removed and the contents of each organ washed into separate evaporating dishes lined with a weighed sheet of nitrogen-free plastic wrapping material.[8] The samples were dried (100°C), weighed and the nitrogen content determined by the Kjeldahl method, using mercuric oxide as the catalyst.

RESULTS

Stomach-emptying. The effects of the different dietary proteins on the rate of

[3] Borden Company, New York.
[4] Nutritional Biochemicals Corporation, Cleveland.
[5] The Drackett Products Company, Cincinnati.
[6] Wilson and Company, Chicago.
[7] Stein Hall Manufacturing Company, Chicago.
[8] Saran Wrap, Dow Chemical Company, Midland, Michigan.

stomach-emptying are shown in figures 1–4. The results are presented both as percentage of ingested nitrogen and as percentage of dry weight recovered at intervals after feeding. Standard errors are not included but the statistical significance

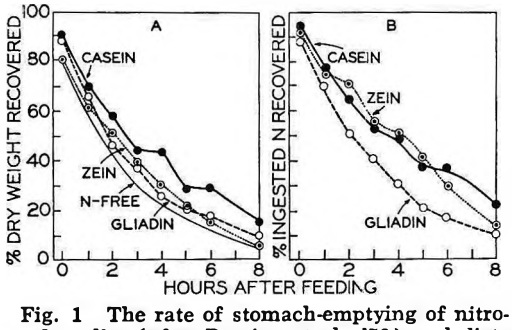

Fig. 1 The rate of stomach-emptying of nitrogen-free diet (after Peraino et al., '59) and diets containing 30% of casein, 30% of gliadin or 30% of zein. A, Percentage of ingested dry weight recovered. B, Percentage of ingested nitrogen recovered.

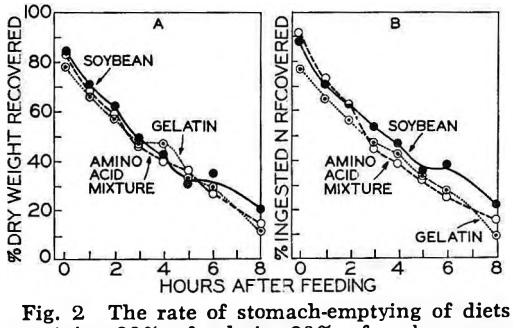

Fig. 2 The rate of stomach-emptying of diets containing 30% of gelatin, 30% of soybean protein or 30% of amino acids. A, Percentage of ingested dry weight recovered. B, Percentage of ingested nitrogen recovered.

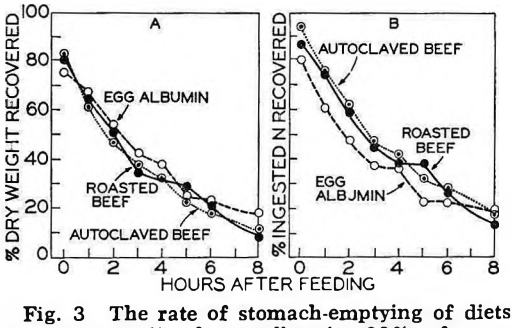

Fig. 3 The rate of stomach-emptying of diets containing 30% of egg albumin, 30% of autoclaved beef or 30% of roasted beef. A, Percentage of ingested dry weight recovered. B, Percentage of ingested nitrogen recovered.

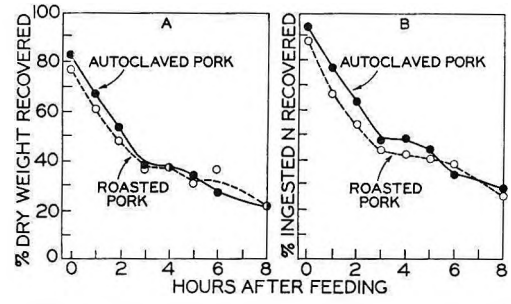

Fig. 4 The rate of stomach-emptying of diets containing 30% of autoclaved pork or 30% of roasted pork. A, Percentage of ingested dry weight recovered. B, Percentage of ingested nitrogen recovered.

of differences between curves is indicated in the text where it was thought necessary. As an example, standard errors of the mean for the points on the curve for the casein diet ranged from $\pm$ 1.0 to $\pm$ 3.3% with the highest values occurring between 3 and 6 hours after feeding.

The curves for the rates of emptying of dry matter from the stomach in figure 1A indicate that the nitrogen-free diet and diets containing gliadin and zein left the stomach at about the same rate and that the diet containing casein emptied more slowly. Dry matter and nitrogen emptied at similar rates when the diet contained casein or gliadin (figs. 1A and B), but dry matter emptied more rapidly than nitrogen when the diet contained zein. At every interval after the first hour the nitrogen of gliadin emptied significantly faster ($P < 0.05$) than that of casein.

The curves in figures 2A and B for the rates of disappearance of dry matter and nitrogen of the diet containing soybean protein were nearly identical with those for the casein diet (fig. 1). The rates of emptying of dry matter of diets containing gelatin, soybean protein or an amino acid mixture were similar. The nitrogen of the gelatin diet emptied faster than that of the casein diet, the differences between the two curves being significant ($P < 0.05$) at hourly intervals zero, 1, 2, 6 and 8. The nitrogen of the amino acid diet emptied more rapidly than that of casein and soybean protein at the later intervals when only a small amount of solids was left in the stomach.

There were no significant differences among the stomach-emptying curves for the dry matter of diets containing egg albumin, autoclaved beef or roasted beef ($P > 0.05$) as shown in figure 3. These three curves fell between those for casein and gliadin (fig. 1A). The nitrogen of the egg albumin diet emptied somewhat faster than that of the beef diets during the early hours. During the first part of the experimental period the pattern for the egg albumin diet resembled that obtained for gelatin (fig. 2) in that the nitrogen emptied more rapidly than the total solids.

The rates at which autoclaved and roasted pork diets emptied from the stomach were not significantly different, as shown in figure 4. The nitrogen of the pork diets emptied more slowly than that of diets containing gelatin, gliadin and egg albumin but at about the same rate as that of casein and soybean proteins.

The stomach-emptying curves obtained with diets containing three levels of gelatin are shown in figure 5. The curves for both dry matter and nitrogen for the 15% gelatin diet are significantly higher than those for the 30% gelatin diet at the zero-, 1-, and 2-hour intervals ($P < 0.05$). The nitrogen of the 50% gelatin diet emptied somewhat more rapidly than the nitrogen of the 30% gelatin diet but at no point were the curves statistically significantly different ($P > 0.05$).

Percentage of nitrogen in stomach contents. In figures 6 and 7 the percentage of nitrogen in the dry matter (N% dry weight) of the stomach contents is plotted against time in hours after feeding. The absolute values for the different diets cannot be compared directly because the diets were not isonitrogenous; nevertheless, the relative values can be compared, and these reveal some interesting patterns.

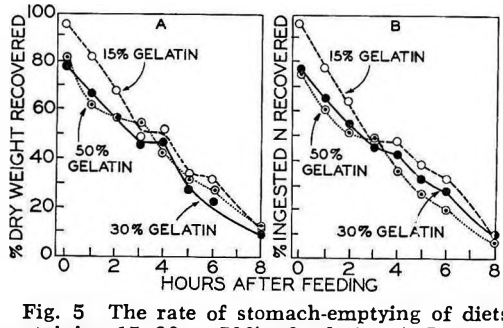

Fig. 5 The rate of stomach-emptying of diets containing 15, 30 or 50% of gelatin. A, Percentage of ingested dry weight recovered. B, Percentage of ingested nitrogen recovered.

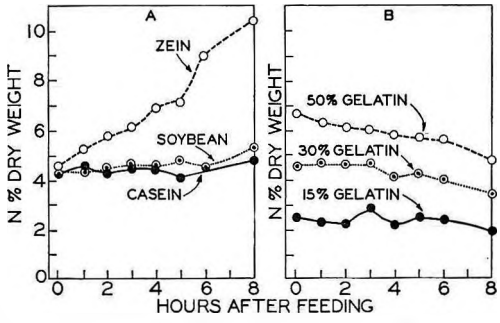

Fig. 6 Change in percentage of nitrogen in stomach contents (N% dry weight = N recovered/dry weight recovered × 100) with time after feeding diets containing 30% of zein, 30% of soybean protein, 30% of casein and 15, 30 or 50% of gelatin.

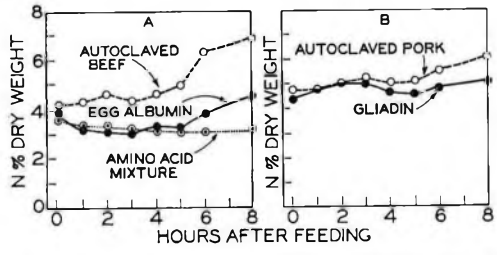

Fig. 7 Change in percentage of nitrogen in stomach contents (N% dry weight = N recovered/dry weight recovered × 100) with time after feeding diets containing 30% of autoclaved beef, 30% of egg albumin, 30% of amino acids, 30% of autoclaved pork or 30% of gliadin.

When the diet contained zein the percentage of nitrogen in the stomach contents increased every hour after the first hour. In contrast, with diets containing soybean protein, casein, gliadin, pork proteins and beef proteins the percentage of nitrogen in the stomach contents remained relatively constant during the earlier hours and increased only during the later hours when the quantity of solids remaining in the stomach was small (figs. 6 and 7). The percentage of nitrogen in the stomach contents after feeding gelatin diets decreased progressively during the entire 8 hours. The patterns for the amino acid diet and the egg albumin diet resemble that for gelatin during the first 5 hours, but during the last three hours there was

no further fall in the curve for the amino acid diet, and that for the egg albumin diet rose considerably.

Intestinal contents. Figures 8 to 10 show the extent of accumulation of nitrogen in the small intestine, reported as milligrams of nitrogen in the contents of the entire small intestine, at intervals after feeding the various diets. The results are presented as milligrams of nitrogen rather than as percentage of ingested nitrogen because the small intestine is in a dynamic state (receiving nutrients and secretions, absorbing nutrients and excreting waste products), and the nitrogen recovered undoubtedly represents nitrogen from several sources.

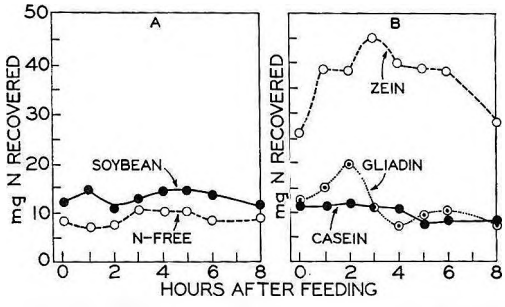

Fig. 8 Milligrams of nitrogen recovered from the entire small intestine of rats fed diets containing no protein, 30% of zein, 30% of casein, 30% of soybean protein or 30% of gliadin.

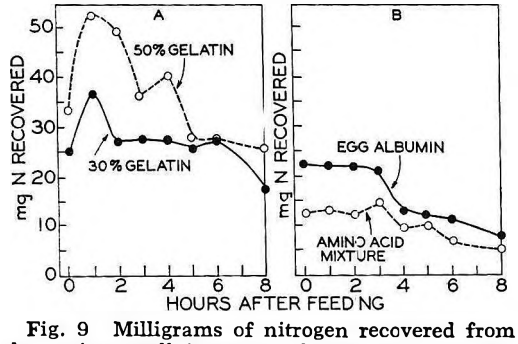

Fig. 9 Milligrams of nitrogen recovered from the entire small intestine of rats fed diets containing 30 or 50% of gelatin, 30% of egg albumin or 30% of amino acids.

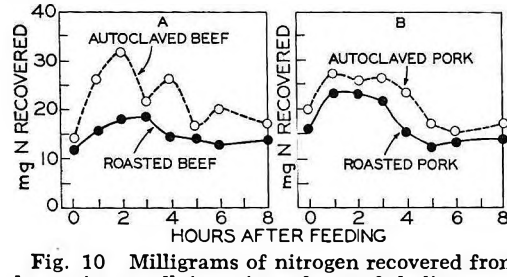

Fig. 10 Milligrams of nitrogen recovered from the entire small intestine of rats fed diets containing 30% of autoclaved beef, 30% of roasted beef, 30% of autoclaved pork, or 30% of roasted pork.

Although there was some increase in the amount of nitrogen in the small intestine when diets containing soybean protein or casein were fed (fig. 8A), the increase over that observed when a nitrogen-free diet was fed was statistically significant for the soybean protein diet ($P < 0.05$) only at the zero- and 1-hour intervals. There was a highly significant ($P < 0.01$) accumulation of nitrogen in the intestine at all hourly intervals for the diet containing zein as compared with the 30% casein diet. A small increase was noted at the second hour after feeding gliadin (fig. 8B) but because of the large variation among the individual values it was not significantly greater than that for casein ($P > 0.05$).

Figure 9A shows the pattern of accumulation of nitrogen in the small intestine when diets containing two levels of gelatin were fed. Feeding the 30% gelatin diet resulted in an accumulation of nitrogen which was significantly greater at all hourly intervals ($P < 0.01$) than that obtained after feeding the 30% casein diet. With 50% of gelatin in the diet the accumulation was even greater. The greatest nitrogen accumulation in the small intestine occurred at the end of the first hour when the diet contained gelatin (fig. 9A) but after the third hour when the diet contained zein (fig. 8B). Figure 9B shows that a small quantity of nitrogen accumulated in the small intestine during the first three hours after feeding the egg albumin diet—significantly higher ($P < 0.05$) than that found after feeding the casein diet, at hours zero, 1, 2 and 3. No increase was noted after feeding the diet containing the amino acid mixture.

The differences between the intestinal nitrogen accumulations after feeding diets containing beef and pork proteins (previously given different heat treatments) are shown in figure 10. The quantity of nitrogen recovered from the small intestine was greater when autoclaved beef was fed than

when roasted beef was fed (statistically significant ($P < 0.05$) at zero, 1, 2, 4 and 5 hours), the time of heat treatment being the same. Feeding autoclaved pork protein resulted in intestinal nitrogen values higher than those obtained after feeding roasted pork proteins, but the only point at which the difference was statistically significant was at the 4th hour ($P < 0.05$). The curve for intestinal nitrogen after feeding roasted pork protein was similar to that obtained after feeding egg albumin except for the zero-hour value.

DISCUSSION

Stomach-emptying and percentage of nitrogen in stomach contents (N% dry weight). Rosenthal and Nasset ('58), Gupta et al. ('58) and others have found that stomach-emptying curves were approximately exponential but in the present study many types of patterns, from nearly exponential (30% gliadin diet) to nearly linear (30% gelatin diet), were obtained. This difference may be due to differences in the experimental animals ("trained" versus "starved rats"), in analytical procedures, in the completeness of the diet, in the level of dietary protein, in the type of dietary carbohydrate and in the amount of diet fed—all of which may influence the rate of stomach-emptying (Peraino et al., '59). Also, in the present study, in agreement with the results of Geiger et al. ('58) but not with those of Dreisbach and Nasset ('54), the amount of nitrogen recovered from the stomach and small intestine after the first hour was always less than the amount fed.

The solubility of a protein appears to influence the rate at which it leaves the stomach. Evidence of this is obtained from measurements of the percentage of nitrogen in the stomach contents at intervals after feeding. This should remain constant if all of the components of the diet empty together, as is common with dextrin-casein diets but not with sucrose-casein diets (Peraino et al., '59); it should increase if the protein remains behind the other components, and decrease if the protein empties most rapidly. The last of these is most difficult to demonstrate because, as the stomach empties, endogenous secretions represent a greater percentage of the residue and the percentage of nitrogen in the stomach contents during the fasting state is always high. However, since absorption of nitrogen from the stomach is negligible (London and Kotchneva, '35; and Karel, '48), even a small decline in the percentage of nitrogen in the stomach contents should be significant.

The percentage of nitrogen in the stomach contents of rats that received zein increased at each hourly interval (fig. 6) indicating that zein, which is quite insoluble, was retained in the stomach longer than the other components of the diet. On the other hand, the percentage of nitrogen in the stomach contents of rats that received gelatin declined steadily (fig. 7)—evidence that gelatin, which is highly soluble, left the stomach more rapidly than the other components of the diet.

Of the other nitrogen sources only egg albumin (undenatured) and the amino acid mixture, both of which are quite soluble, showed trends similar to that for gelatin during the first 5 hours. The percentage of nitrogen in the stomach contents remained quite constant during the first few hours after feeding gliadin, soybean protein, casein, pork proteins and beef proteins, indicating that these proteins emptied at about the same rates as the other dietary components. The upward trend shown by some proteins after the 5th hour may be due to the movement of the carbohydrate from the stomach ahead of the protein or may be just a reflection of continuous secretion of endogenous nitrogen which then becomes a greater percentage of the material remaining in the stomach.

The observation that the percentage of nitrogen in the stomach contents decreased (fig. 8) more rapidly when the percentage of gelatin in the diet was raised can be explained in the following manner. More fluid was present in the stomachs of rats fed higher levels of gelatin (probably due to the higher osmotic pressure of gelatin), and the gelatin being soluble could thereby move out ahead of the other components of the diet. A similar solubility effect was found with a casein-sucrose diet by Peraino et al. ('59), but in the latter case the carbohydrate, which was the more soluble component, emptied most rapidly. These observations indicate that it is not advis-

able to generalize from results with a single protein about the effect of the level of dietary protein on the stomach-emptying pattern.

In an occasional experiment variations among individual animals were large. There appeared to be no correlation between the range of variation and the length of training time (between 2 to 3 weeks), adaptation to the diet or any known environmental factor. Other factors, such as the emotional state of the animal, which influence stomach-emptying (Thomas, '57) may account for some of these effects.

Intestinal contents. The type of dietary protein had a profound effect upon the quantity of nitrogen recovered from the small intestine. When diets containing soybean protein, casein, gliadin or the amino acid mixture (figs. 8 and 9) were fed, very little accumulation of nitrogen was observed in the small intestine over that found after feeding a nitrogen-free diet. This accumulation is probably of little significance since Peraino et al. ('59) found that the amount of nitrogen in the contents of the small intestine increases very little when the casein content of the diet is increased from 15 to 50%. Similar nitrogen values for the intestinal contents of rats have been reported by Rosenthal and Nasset ('58) and Geiger et al. ('58). These observations lead to the conclusion that for the accumulation of nitrogen in the small intestine to be of significance in studies of protein digestibility, it must be greater than that found after feeding casein diets. For this reason most of the results have been compared with values obtained after feeding the 30% casein diet.

The greatest amount of nitrogen was recovered from the small intestines of rats receiving zein. Gupta et al. ('58) also found some accumulation of nitrogen in the small intestine of "trained" rats when a zein diet was fed. Zein is known to be poorly digested; Geiger et al. ('52) reported that some of the peptide bonds in zein are not broken and that nearly all of the valine is excreted in the feces. How much of this effect is due to its insolubility and how much to resistant peptide bonds remains to be shown.

The digestibility of gelatin, as determined by nitrogen balance studies, is about 95% (Mitchell and Block, '46). Nevertheless, there was also a considerable accumulation of nitrogen in the small intestines of rats fed diets containing 30 or 50% of gelatin, with the largest accumulation occurring at the end of the first hour (fig. 9). This suggests that hydrolysis of some of the peptide bonds of gelatin may be slow or that the rate of absorption of the unbalanced amino acid mixture arising from gelatin may be slow.

In general the results of the present investigation agree quite well with those of London and Rabinowitsch ('11) who investigated the rate of digestion of various proteins in dogs prepared with various gastrointestinal fistulas. They found after examination of the products of digestion that hydrolysis was least with elastin, greater with gelatin and gliadin, and greatest with blood and meat proteins. An accumulation of peptides in the small intestine could account for the observation that more liquid accumulated in the intestines of rats fed the gelatin diets than in the intestines of those fed other diets; and for the observation that rats fed diets containing high levels of gelatin frequently develop diarrhea.

Somewhat more nitrogen accumulated in the small intestine when the diet contained egg albumin than when it contained casein. The greatest accumulation occurred immediately after the feeding period, a pattern similar to that obtained when the diet contained gelatin. Since undenatured egg albumin is readily soluble it may move into the intestine fairly rapidly until it is denatured, then enter more slowly (fig. 9B). This would also explain why egg albumin left the stomach more rapidly than the other dietary components during the first part of the experiment, then more slowly during the last part (fig. 7A).

The greatest amount of nitrogen accumulated in the small intestine while dietary nitrogen was emptying most rapidly from the stomach. For example, the nitrogen of the gelatin and egg albumin diets left the stomach most rapidly during the first hour and during this period the greatest accumulation of nitrogen was found in the intestine; zein was held in the stomach

longer and the greatest nitrogen accumulation occurred in the small intestine at the end of the third hour.

Little can be concluded about the effect of heat treatment on the digestibility of meat proteins from this study but the results (fig. 10) indicate that meat which had the most severe heat treatment was rendered least digestible. Wheeler and Morgan ('58) showed that pork autoclaved for 4 hours supported a slower growth rate in rats than raw pork, and Lushbough et al.[9] showed that increasing the heat treatment of meat reduced the availability of lysine.

SUMMARY

The rate of disappearance of nitrogen from the stomach and the small intestine of "trained" rats consuming purified diets (containing all the proximate principles) containing 30 or 50% of gelatin, and 30% of casein, soybean protein, zein, gliadin, egg albumin, beef proteins, pork proteins or an amino acid mixture has been determined.

The slowest rate of stomach-emptying was noted when the diet contained 30% of pork proteins whereas the most rapid rate of stomach-emptying was found with the 30% gliadin diet. The shapes of the stomach-emptying curves varied from almost exponential (gliadin diet) to nearly linear (gelatin diet).

The percentage of nitrogen in the stomach contents (mg N/mg dry weight × 100) remained nearly constant during the early hours of the experimental period studied when the diet contained casein, soybean protein, beef proteins, pork proteins, or gliadin; decreased slightly when the diet contained gelatin or egg albumin, and possibly for that containing amino acids; and increased greatly when the diet contained zein.

More nitrogen accumulated in the small intestines of rats fed diets containing zein and gelatin than in the intestines of those fed casein; a small accumulation was found when the diet contained egg albumin, pork or beef proteins and no accumulation above that for rats fed casein was observed when the diet contained the amino acid mixture, soybean protein or gliadin. Heat treatment of meat proteins appeared to render the meat less digestible.

The conclusion is drawn that it is not advisible to generalize from results obtained with a single protein about the effect of the protein content of the diet on the rate of stomach-emptying and intestinal accumulation of nitrogen.

[9] Lushbough, C. H., M. R. Chutkow, B. S. Heller and B. S. Schweigert 1959 The effect of heat treatment on availability of lysine from meat. Federation Proc., *18:* 534 (abstract).

LITERATURE CITED

Dreisbach, L. S., and E. S. Nasset 1954 Absorption of carbohydrate and protein as affected by feeding cornstarch, banana and glucose. J. Nutrition, 53: 523.

Geiger, E. 1951 Extra-caloric function of dietary components in relation to protein utilization. Federation Proc., *10:* 670.

Geiger, E., G. W. Courtney and L. E. Geiger 1952 The physiological availability of some essential amino acids present in zein, in the acid and in the enzymatic hydrolyzate of zein. Arch. Biochem. Biophys., *41:* 74.

Geiger, E., L. E. Human and M. J. Middleton 1958 Nitrogen content of the gastrointestinal tract of rats during the absorptive period. Proc. Soc. Exp. Biol. Med., 97: 232.

Gupta, J. D., A. M. Dakroury and A. E. Harper 1958 Observations on protein digestion *in vivo*. I. Rate of disappearance of ingested protein from the gastrointestinal tract. J. Nutrition, 64: 447.

Harper, A. E. 1959 Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance. Ibid., *68:* 405.

Harper, A. E., and D. A. Benton 1956 Observations on some nutritional factors that influence the lipotropic activity of methionine. Biochem. J., 62: 440.

Harper, A. E., and U. S. Kumta 1959 Amino acid balance and protein requirement. Federation Proc., *18:* 1136.

Karel, L. 1948 Gastric absorption. Physiol. Rev., *28:* 433.

London, E. S., and A. G. Rabinowitsch 1911 Zum Chemismus dere Verdauung und Resorption in tierischen Korper. XL. Mitteilung. Der Grad des Abbaues von verschiedenen Eiweissarten in Lumen des Magendarmkanals. Ztschr. Physiol. Chem., *74:* 305.

London, E. S., and N. Kotchneva 1935 The form of absorption of protein from the alimentary tract and further fate of the products of absorption. Arch. sci. biol. (USSR) 37: 3. [Chem. Abs., *30:* 7183 (1936)].

Mitchell, H. H. 1948 The biological utilization of proteins and protein requirements. In Proteins and Amino Acids in Nutrition, ed., M. Sayhun. Rheinhold Publishing Company, New York, p. 46.

Mitchell, H. H., and R. J. Block 1946 Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem., *163:* 599.

Nasset, E. S. 1957 Role of the digestive tract in the utilization of protein and amino acids. J.A.M.A., *164:* 172.

Nasset, E. S., P. Schwartz and H. V. Weiss 1955 The digestion of protein *in vivo*. J. Nutrition, *56:* 83.

Peraino, C., Q. R. Rogers, M. Yoshida, M. Chen and A. E. Harper 1959 Observations on protein digestion *in vivo*. II. Dietary factors affecting the rate of disappearance of casein from the gastrointestinal tract. Canad. J. Biochem. Physiol., *37:* 1475.

Rosenthal, S., and E. S. Nasset 1958 Gastric emptying and intestinal absorption of carbohydrate and protein as influenced by the nature of the test meal. J. Nutrition, *66:* 91.

Thomas, J. E. 1957 Mechanics and regulation of gastric emptying. Physiol. Rev., *37:* 453.

Wheeler, P., and A. F. Morgan 1958 The absorption by immature and adult rats of amino acids from raw and autoclaved fresh pork. J. Nutrition, *64:* 137.

Nutrition of Salmonoid Fishes

VIII. INDISPENSABLE AMINO ACIDS FOR SOCKEYE SALMON[1]

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The indispensable amino acid requirements of chinook salmon *(Oncorhynchus tshawytscha)* have recently been reported (Halver et al., '57) and additional studies have established gross protein requirements at two water temperatures (DeLong et al., '58). An amino acid test diet has also been described which maintained chinook salmon fingerlings at a reasonable growth rate for at least 14 weeks (Halver, '57b). This concentrated work using one species of salmon has not been applied to other salmonoid fishes. In the present study another important species of salmon, the sockeye salmon *(O. nerka)*, has been tested with these diets under these experimental feeding conditions to confirm the validity for salmon of the indispensable nature of the 10 amino acids included in the protein component of the test diets. Close attention was directed toward the arginine-deficient fish to confirm the general requirement for this amino acid in this family of experimental animals. Similar emphasis was placed on the tryptophan-deficient group because of the slightly reduced nicotinic acid complement of the vitamin component in the experimental ration.

EXPERIMENTAL

The methods of diet preparation and general feeding techniques have been reported previously (Halver, '57a, b; Halver et al., '57). Major quantitative modifications in the nitrogen and vitamin components of the diet were introduced on the basis of the protein requirement studies with chinook salmon (DeLong et al., '58) and the vitamin studies with silver salmon *(O. kisutch)* (Coats and Halver, '58). In the deficient diets α-cellulose flour replaced, on an equal weight basis, the amino acid deleted from the basal ration (table 1).

TABLE 1
Basal ration

Constituent	Amount
	gm
L-Arginine·HCl	3.6
L-Histidine·HCl·H_2O	1.8
L-Isoleucine	2.9
L-Leucine	4.3
L-Lysine·HCl	3.6
L-Methionine	1.4
L-Threonine	2.9
L-Tryptophan	0.7
L-Tyrosine	2.9
L-Valine	2.9
L-Threonine	1.8
Glycine	5.3
L-Alanine	2.5
L-Aspartic acid	3.6
L-Cystine	0.5
L-Glutamic acid	5.7
L-Proline	3.6
White dextrin	25.0
Corn oil	5.0
Cod liver oil	2.0
Carboxymethylcellulose	10.0
Mineral mix[1]	4.0
α-Cellulose + vitamins[2]	4.0
Water	100

[1] Mineral mix the same as reported previously (Halver, '57a).
[2] Contains (in milligrams): thiamin·HCl, 5; riboflavin, 20; pyridoxine·HCl, 5; choline chloride, 500; nicotinic acid, 75; Ca pantothenate, 50; inositol, 200; biotin, 0.5; folic acid, 1.5; ascorbic acid, 100; menadione, 4; α-tocopheryl acetate, 40; cyanocobalamine, 0.01.

Feeding trials were conducted in covered 10-gallon glass aquariums supplied with a constant flow of deep well water, heated to 11°C. A representative random sample of 10,000 eyed sockeye salmon eggs, obtained from the Leavenworth National Hatchery, Leavenworth, Washington, were hatched and reared to approximately 0.2 gm in constant temperature water (11°C).

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[1] Presented at the meeting of the American Institute of Nutrition, Chicago, 1960.

Since only 12 aquariums were available for experimental use, the feeding trials were run in two sections of 10 individual lots of deficient fish compared with duplicate controls. After the groups had been adapted for two weeks to the basal ration and to the aquarium environment, all dead fish were replaced and the specific amino acid-deficient diets were offered three times daily. The fish were fed a slowly sinking diet expelled through a garlic press at the surface of the water. Diets were fed only as long as the fish accepted them and feeding was stopped as soon as any portion of the food reached the bottom of the aquarium. Apparatus common to more than one lot was cleaned and disinfected between aquariums to minimize transfer of disease organisms or food particles. Fish were fed three times daily, 6 days weekly on a rigid schedule, (8 A.M., 1 P.M. and 4 P.M.). Aquariums were partially cleaned daily without removing the fish and were drained and thoroughly cleaned during the absence of the fish at the weighing period. The entire population was weighed biweekly by transferring drained fish to a small tared container resting on a solution balance which held only enough water to maintain the fish during the weighing period.

With only twelve 10-gallon aquariums available for the experiments, only 10 experimental deficient and two control lots could be compared at one time. Therefore, 5 indispensable and 5 dispensable amino acids (for chinook salmon) were compared with two controls. After 6 weeks of feeding, the 5 dispensable amino acid-deficient groups of fish exhibiting comparable growth with the controls were discarded; the 5 indispensable amino acid-deficient groups were divided evenly and half of each lot was placed in the vacant aquariums. Fortunately, in every case, little or no growth was observed during this 6-week period in those groups of fish deficient in one of the selected indispensable amino acids. Conversely, each lot of fish fed the dispensable amino acid-deficient rations grew at a rate comparable with the controls. During the last segment of each experimental period, half the population continued to receive the indispensable amino acid-deficient diet and the other half the complete amino acid ration. Larger fish imposed space limitations during the second section of the experiments. The average initial weights of the fish available for methionine, phenylalanine, valine, tryptophan, cystine and glycine tests were approximately 1.2 gm and a correspondingly smaller number of individuals (100) were housed in each aquarium. The same general technique of feeding for 6 weeks was followed. Growth or lack of growth in each group of fish was observed, with subsequent discard of the nonessential amino acid-deficient lots. Again significant differences were observed in the response to the various deficient rations. The indispensable amino acid-deficient lots were then divided into equal sublots and growth was measured when the missing amino acid was replaced in each ration.

RESULTS

Arginine, histidine, isoleucine, leucine and lysine were found to be indispensable for normal growth of sockeye salmon in the first group of experiments. No significant growth was observed in any group fed diets missing one of these amino acids, indicating negligible biosynthesis (table 2). Within two weeks a definite loss in appetite was noted. Feeding continued until the end of the 12-week experimental period, however, and total mortality in the indispensable amino acid-deficient groups approximated 40% of the original population compared with a loss of approximately 15% in the control or nonessential amino acid-deficient groups. At the end of the 6th week of feeding, the fish in each of the 5 indispensable amino acid-deficient lots were split into two subgroups. Subgroup 1 continued to receive the same amino acid-deficient ration and subgroup 2 was assigned the basal diet containing all 17 crystalline amino acids. Fish in subgroup 2 showed an immediate and substantial growth response to the complete diet. The fish in subgroup 1, fed the deficient diet, continued to show a curbed food intake and a loss of activity throughout the 12 weeks. The positive growth response observed when the missing amino

TABLE 2

Average data for sockeye salmon fed various diets

Diets	Initial weight	Av. weight 6th week	Av. weight 12th week
	gm	*gm*	*gm*
Arginine-deficient	0.216	0.203	0.261
Basal		0.206	0.433
Histidine-deficient	0.213	0.256	0.257
Basal		0.244	0.348
Isoleucine-deficient	0.216	0.248	0.272
Basal		0.241	0.378
Leucine-deficient	0.200	0.201	0.225
Basal		0.198	0.393
Lysine-deficient	0.220	0.238	0.254
Basal		0.227	0.343
Control group 1	0.221	0.367	0.777
Control group 2	1.270	2.227	
Methionine-deficient	1.345	1.619	1.757
Basal		1.524	2.564
Phenylalanine-deficient	1.325	1.576	2.043
Basal		1.619	2.750
Valine-deficient	1.220	1.446	1.446
Basal		1.305	2.074
Threonine-deficient	1.285	1.425	1.385
Basal		1.427	2.244
Tryptophan-deficient	1.117	1.239	1.314
Basal		1.467	2.279
Cystine-deficient	1.295	2.126	
Glycine-deficient	1.200	1.647	
Alanine-deficient	0.213	0.331[1]	
Aspartic-deficient	0.216	0.344[1]	
Glutamic-deficient	0.205	0.344[1]	
Tyrosine-deficient	0.204	0.322[1]	
Proline-deficient	0.212	0.337[1]	

[1] This group terminated after 5 weeks.

acid was replaced in the ration can be seen in the typical growth curve shown in figure 1.

Alanine, aspartic acid, glutamic acid, proline and tyrosine were found to be dispensable amino acids for normal growth of sockeye salmon under the conditions of this experiment (table 2). Fish receiving these diets fed actively and gained weight throughout the course of the feeding trial. Mortality in each of these test groups was as low as or lower than that of the control lot fed the complete amino acid complement for the protein component of the ration. Growth of the test group paralleled closely that of the control group and at the time of the division of the indispensable amino acid groups of fish, no abnormal clinical signs of any deficiencies could be observed. Due to lack of space and since these lots were growing so well, the trial was discontinued when the original diet preparation had been exhausted after 5 weeks of feeding.

Methionine, phenylalanine, valine, tryptophan and threonine were found to be indispensable for normal growth of sockeye salmon in the second set of experiments. Because of the aquarium size it was necessary to use lots of 100 fish, averaging approximately 1.2 gm each. Curbed food intake was observed within two weeks and a general loss of activity as the experiment progressed in each lot of fish deficient in one indispensable amino acid. The control lots and the cystine- and glycine-deficient groups ate avidly and failed to show the appearance of any specific or general deficiency syndrome. It

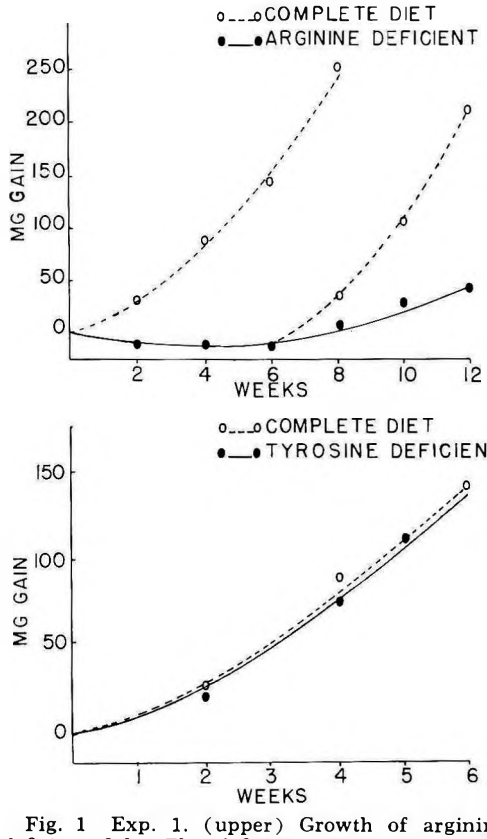

Fig. 1 Exp. 1. (upper) Growth of arginine-deficient fish. The deficient group was divided after feeding the deficient diet for 6 weeks and the missing amino acid was replaced in one of the two subgroups; (lower) growth of tyrosine-deficient fish. No difference was discernible between tyrosine-deficient and control lots of salmon.

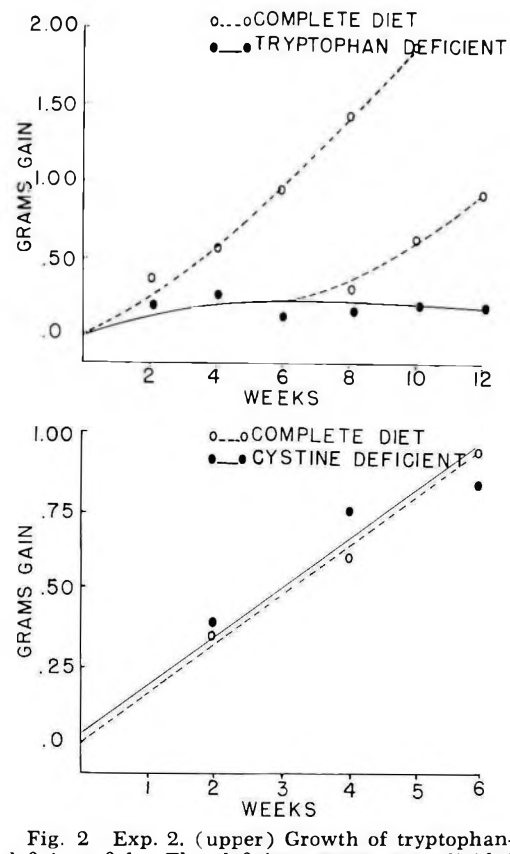

Fig. 2 Exp. 2. (upper) Growth of tryptophan-deficient fish. The deficient group was divided after feeding the deficient diet for 6 weeks when the missing amino acid was replaced in one of the two subgroups; (lower) growth of cystine-deficient fish. No difference was discernible between cystine-deficient and control lots of salmon.

was necessary to discard the cystine- and glycine-deficient lots after 6 weeks of feeding to make room for the recovery subgroups of the indispensable amino acid-deficient lots. Mortality throughout the second set of experiments was low and these larger fish were much easier to handle in the experimental facilities. Results of growth are summarized in the last half of table 2 and figures 1 and 2.

A specific deficiency syndrome for tryptophan was observed after only 4 weeks of feeding. Pronounced scoliosis with some lordosis was noted in approximately 20% of the population (fig. 3). This condition continued to develop in the deficient lot until the end of the 12–week feeding period when over 50% of the fish had become deformed. Little mortality occurred, with only one fish dying with this condition in the deficient lot after the division. Extreme care was exercised in dividing the affected fish equally in the two sublots to measure response when the missing amino acid was replaced in the ration. In every case, the scoliosis and lordosis disappeared within two weeks after replacing tryptophan in the ration; whereas in the tryptophan-deficient fish the condition continued to develop until, at the end of the experimental feeding trial, over half the population had definite symptoms of this syndrome. Except for the larger size in this second section of experiments, the individual lots of fish responded as observed in the first group of experiments.

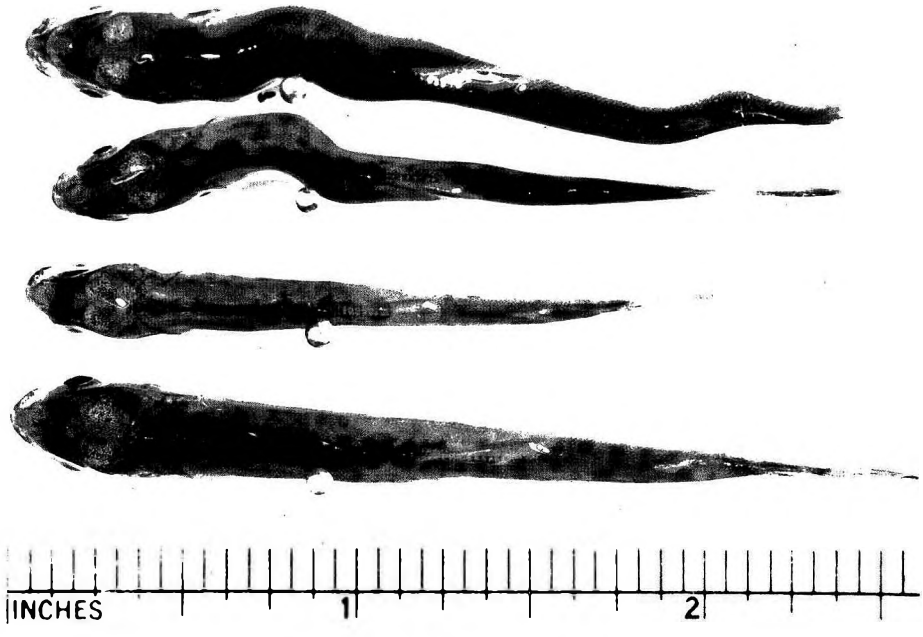

Fig. 3 Tryptophan-deficient fish. The upper two fish show pronounced scoliosis following deletion of tryptophan from the ration for 21 to 28 days. The two lower fish show the condition reduced in 7 to 14 days after tryptophan was added to the deficient ration.

Some difference in the growth response was observed between the glycine-deficient and the control lots but it was not possible with the facilities available to continue the glycine-deficient group beyond the 6–week period without sacrificing the study of one of the 5 specifically indispensable amino acids included in this section of the feeding trial.

DISCUSSION

Seventeen L-amino acids were tested at levels considered to furnish an excess over minimum requirements for reasonable growth (Halver, '57b). The water temperature for this study was intermediate between those temperatures used to establish the protein requirements of chinook salmon (DeLong et al., '58). Food efficiency and the protein efficiency ratio could not be determined accurately because of the soluble nature of many of the components in the prepared ration. Mortality was a significant factor in the first set of experiments with those lots of fish deficient in an indispensable amino acid exhibiting a loss approaching 50% of the original population. Even in the control lots and in those groups fed dispensable amino acid-deficient diets, the mortality rate was appreciable. During the weighing period, the entire population of each aquarium was flushed through a one-inch-diameter drain cut in the glass bottom and the force of the water may have caused harm and resulted in an increase of mortality with these small fish. Handling fish with average weights of 0.2 to 0.3 gm presented challenging problems and extreme care was necessary in feeding, very slowly, minute particles of the ration to induce them to feed.

Subsequent cleaning operations and removal of waste products required additional care and patience, since the siphons used to clean the aquariums daily readily engulfed fish and discharged them maimed or dead in the drain. These considerations increased the probability of higher mortality rates than in the second set of experiments when larger fish were handled with

relatively no loss during the 12-week experimental period. For these reasons, mortality was not considered as a diagnostic parameter in interpreting the response to a particular treatment, and tentative conclusions were drawn only on the actual growth response when a specific ration was fed under carefully controlled conditions to large groups of fish. In the second set of experiments, a pronounced scoliosis developed in the tryptophan-deficient group of sockeye salmon. The symptoms became apparent after only 4 weeks of feeding the deficient ration, in contrast with the lack of any specific symptom in tryptophan-deficient chinook salmon fingerlings after 10 weeks of feeding a similar ration (Halver et al., '57). In the chinook salmon study, slightly higher levels of nicotinic acid were included in the vitamin component of the ration and the amino acid component was fed at approximately 70% of the dry ration. In subsequent work with silver salmon (Coats and Halver, '58) and in further studies of the chinook salmon vitamin requirements,[2] adequate growth response was observed when the nicotinic acid was fed at 750 mg per kg of solids.

The increased carbohydrate component in this current ration would require increased quantities of nicotinic acid to metabolize if the critical interrelationships between the requirements for nicotinic acid and the carbohydrate intake of other animals are applicable in fish metabolism. It is interesting to speculate upon the striking similarity between the scoliosis and lordosis observed in the tryptophan-deficient group of sockeye salmon and the characteristic lordosis and scoliosis observed in survivors of sockeye salmon "virus" disease (Wood and Yasutake, '56). Perhaps an interrelationship exisits between tryptophan and/or nicotinic acid metabolism and the relative severity of these virus attacks and it may be possible to study the site and metabolic intermediates in virus-infected sockeye salmon now that tryptophan-controlled rations are possible, to induce tryptophan-deficiency symptoms.

In addition to the 17 amino acids tested, serine, by elimination, was also a dispensable amino acid for normal growth for sockeye salmon since it was not included in the amino acid component of the ration. Although glycine-deficient fish were discarded in favor of the major indispensable amino acid studies, some lack of complete growth response was noted after 6 weeks of feeding the glycine-deficient ration. These individual fish averaged 1.65 gm compared with 2.26 gm for the control lots. Unfortunately, space did not permit continuing the experiment with this group of deficient fish and a more extended feeding trial will be necessary before the complete dispensability of glycine can be verified under these experimental conditions.

All dead fish found in both sets of these experiments were examined carefully but none of the commonly occuring fish pathogens were observed. The water supply was pumped from a deep well under closed conditions through a heat exchanger directly into the aquariums to prevent inadvertent contamination with fish pathogens. For this reason, and from the lack of observation of disease organisms, macroscopically and microscopically by pathologists, it seemed obvious that the differential growth of lots fed the different rations was due to the particular nutritional treatment involved.

SUMMARY

Sockeye salmon fingerlings were adapted to and fed a test ration containing 17 L-amino acids as the nitrogen source. Specific amino acid-deficient diets were prepared by deleting one amino acid from the basal ration and replacing it with α-cellulose flour. Growth with these diets was compared with that obtained with the basal ration. On the basis of the results, sockeye salmon fingerlings required arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine for normal growth. No partial biosynthesis of any of these amino acids was observed. In contrast, alanine, aspartic acid, cystine, glutamic acid, proline and tyrosine were not required for growth and

[2] Halver, J. E. 1960 Vitamin and amino acid requirements of salmon. Fifth International Congress of Nutrition, Washington, D.C. (abstract no. 191).

may be considered dispensable under the experimental conditions used. By inspection hydroxyproline and serine can also be classified dispensable.

ACKNOWLEDGMENTS

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LITERATURE CITED

Coates, J. A., and J. E. Halver 1958 Water soluble vitamin requirements of silver salmon (*O. kisutch*). U.S. Fish and Wildlife Service Special Scientific Report, Fisheries, no. 281.

DeLong, D. C., J. E. Halver and E. T. Mertz 1958 Nutrition of salmonoid fishes. VI. Protein requirements of chinook salmon at two water temperatures. J. Nutrition, 65: 589.

Halver, J. E. 1957a Nutrition of salmonoid fishes. III. Water-soluble vitamin requirements of chinook salmon. Ibid., 62: 225.

——— 1957b Nutrition of salmonoid fishes. IV. An amino acid test diet for chinook salmon. Ibid., 62: 245.

Halver, J. E., D. C. DeLong and E. T. Mertz 1957 Nutrition of salmonoid fishes. V. Classification of essential amino acids for chinook salmon. Ibid., 63: 95.

Wood, E. M., and W. T. Yasutake 1956 Histopathologic changes of a virus-like disease of sockeye salmon. Trans. Microscop. Soc., 75(1): 85.

Pathology of Arginine Deficiency in the Chick[1]

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During the early work which established arginine and glycine as essential dietary constituents for the chick, it was observed that the deficient chick developed a muscular dystrophy or paralysis characterized by a high-stepping, stilted gait (Hegsted et al., '41; Almquist et al., '41). The paralysis was accompanied by a subnormal content of muscle creatine. Hegsted et al. ('41) reported that the muscles, although underdeveloped, were histologically normal but that there were lesions in the spinal cord. However, the detailed results of the histological study were not presented. Feathering was abnormal when arginine and glycine were inadequate; the barbs were poorly developed and the shaft was brittle and easily broken.

In a study of arginine deficiency in the chick, Jungherr et al. ('58) observed cytopathologic changes in the liver cord cells characterized by hypertrophy of the nucleolus. None of the other visceral organs examined showed consistent gross or microscopic change although spinal cord was apparently not studied.

The observations made during the present investigation offer an explanation for the ataxia and muscle attenuation as well as for the bone deformity commonly associated with arginine deficiency.

EXPERIMENTAL

Single-Comb White Leghorn cockerels from the University flock were placed in electrically-heated batteries at hatching and supplied feed and water ad libitum. The basal diet was similar to that described by Savage and O'Dell ('60) except that it contained 1.5% of glycine. The major dietary constituents (in per cent) were: water-washed casein, 35; glucose hydrate, 43.8; cellulose,[3] 3.0; salts (Richardson and Hogan, '46), modified to supply 60 ppm of zinc, 5.0; $CaCO_3$, 1.0; soybean oil, 10.0; DL-methionine, 0.5; glycine, 1.5; choline Cl, 0.2; antioxidant,[4] 0.0125. By microbiological analysis the basal diet contained 1.2% of arginine. A complete vitamin supplement[5] was supplied and the dietary variations included supplementation with L-arginine HCl at levels which supplied 0.6 and 1.2% of the free base, with creatine hydrate as shown in table 1.

Tissues for histological examination were taken from three chicks fed each of three diets used in a previous study (Savage and O'Dell, '60). The chicks selected were typical of 15 fed the basal diet, 55 receiving the basal diet plus 0.6% of arginine and 44 fed the basal plus 1.2% of arginine. The chicks receiving the basal diet developed severe gross symptoms of arginine deficiency as shown by poor growth and feathering, ataxia and leg weakness. The diet supplemented with 1.2% of arginine was adequate by all criteria applied. After consuming the respective diets for 4 weeks the chicks were sacrificed and samples of the skin, brain, spinal cord, sciatic nerve, gonads, liver, kidney, adrenal, spleen, thymus, bursa of Fabricius, heart, skeletal muscle and tibia were taken for histological study. These tissues were fixed in Bouin's solution, sec-

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[2] Present address: Department of Animal Husbandry and Nutrition, Auburn University, Auburn, Alabama.

[3] Solka Floc, Brown Company, Chicago.

[4] Santoquin (1, 2-dihydro-6-ethoxy-2, 2, 4-trimethylquinoline), Monsanto Chemical Company, St. Louis, Missouri.

[5] The authors gratefully acknowledge gifts of vitamins from Merck, Sharp and Dohme, Inc., American Cyanamid Company and Hoffman-La-Roche, Inc., of creatine from Allied Chemical Corporation and of arginine from General Mills, Inc.

tioned, and stained with hematoxylin and eosin in the routine manner. Nerve tissues for myelination studies were fixed in 10% neutral formalin containing 1% of calcium chloride and 1% of cadmium chloride. They were mordanted in 3% potassium dichromate for 48 hours, routinely processed for paraffin sections and stained with Sudan black B, according to the method of the Armed Forces Institute of Pathology ('57). Ten per cent neutral formalin was used to fix tissues for study of ribonucleic acid (RNA) and deoxyribonucleic (DNA). The methyl-green pyronin and ribonuclease method of Brachet was used for RNA and the method of Feulgen with minor modifications was used for DNA (Pearse, '53). Liver and muscle tissues were examined for glycogen content by use of Best's carmine method (Armed Forces Institute of Pathology, '57). Luxol Fast Blue B, silver and gallocyanin stains were used for nerve cell study. Bone was decalcified in formic acid-sodium formate solution and stained with hematoxylin and eosin in the routine manner.

Muscle for creatine determination was removed from the femur, immediately frozen and held in the frozen state until analyzed. Creatine was determined by the method of Rose et al. ('27).

RESULTS

Muscle creatine analysis. Inasmuch as low muscle creatine is associated with arginine deficiency, and creatine is essential for normal muscle function, it seemed possible that the ataxic gait was caused by an abnormally low creatine content. To test this hypothesis, muscle tissue from chicks exhibiting symptoms of muscle paralysis and from apparently normal animals fed the same diet were analyzed for creatine. The results are summarized in table 1. Arginine was supplied at two levels and graded levels of creatine were added to the suboptimal level of arginine. When the diet supplied about 1.8% of arginine the muscle creatine averaged 3.01 mg per gm and 80% of the chicks showed gross symptoms of muscle paralysis. The muscle creatine content increased with increasing levels of dietary creatine up to 0.5% of the diet. This amount of dietary creatine gave a maximal muscle creatine content but did not prevent muscle paralysis. When the diet contained 2.4% of arginine there was no evidence of muscle paralysis but the creatine content of the muscle was not maximal. Furthermore, leg muscles of the chicks that showed gross symptoms of paralysis contained as much creatine as the average, although their body weights were grossly inferior. It seems unlikely that the ataxia is directly related to the creatine content of the muscle.

Histopathologic observations. Alterations were observed in the feather, bone, striated muscle, nerve, liver and lymphoid tissues. The other tissues examined showed no changes of significance. Lesions were observed in birds of all groups except those receiving 1.2% of arginine supplementa-

TABLE 1

Relation of muscle creatine and paralysis to dietary arginine and creatine

Basal diet + supplements		Average weight at 4 weeks	Average muscle creatine[1]	Chicks with muscle paralysis[2]		
Arginine	Creatine + H_2O				Weight	Muscle creatine
%	%	*gm*	*mg/gm*	%	*gm*	*mg/gm*
0.6	—	236(10)[3]	3.01 ± 0.10[4]	80	196	3.05
0.6	0.05	320(5)	3.25 ± 0.06	40	216	3.30
0.6	0.15	271(5)	3.48 ± 0.08	60	213	3.55
0.6	0.30	388(5)	3.50 ± 0.11	20	266	3.75
0.6	0.50	368(5)	3.73 ± 0.09	20	272	3.86
0.6	1.00	357(6)	3.75 ± 0.08	17	216	3.65
1.2	—	410(5)	3.23 ± 0.03	0	—	—
1.2	1.00	405(5)	3.71 ± 0.06	0	—	—

[1] Leg muscle creatine expressed as milligrams of creatinine per gram wet weight.
[2] Chicks that showed a stilted gait and muscular dysfunction.
[3] Figures in parentheses indicate number analyzed.
[4] Standard error of the mean.

tion. The degree of severity of lesions followed roughly the pattern of muscular paralysis as shown in table 1.

Bone. Chicks severely deficient in arginine typically showed a bone deformity in which the legs splayed out from the hock joint. This resulted in a characteristic stance which was traced to a defect in development of the tibia. In its distal third it showed a flattening and a lateral rotation which directed the condyle from its normal axis (fig. 1).

Microscopically, bone growth was greatly depressed or interrupted. The epiphyseal disc was narrow and the bony trabeculae were small and scanty. This resulted in rather large tunnels among the trabeculae. These spaces contained what appeared to be normal hemopoietic elements. Osteogenesis was reduced resulting in striking changes in the bony shaft. Numerous areas that contained a degenerate, fibrillar bony matrix were observed (figs. 2 and 3). Intercellular ground substance was absent in these areas. Osteocytes showed varying degrees of pyknosis and degeneration. Some areas were completely devoid of osteocytes and many of the osteocytes in the zone of appositional bone growth were spindle shaped and appeared to be inactive.

Muscle. Arginine-deficient chicks showed atrophy of muscle fibers which was most pronounced in the muscle groups of the legs. An occasional atrophic fiber was observed in the wing muscles. The fibers were smaller than normal, kinked or curly in appearance, and lacking in the characteristic striations. In some areas the muscles fibers had atrophied, leaving only the connective tissue stroma which contained an abnormal number of small dark nuclei (figs. 4 and 5). Muscle tissue of the deficient birds showed only traces of glycogen as compared with those that received an arginine supplement.

Nerve tissue. Motor neurons of the cerebral cortex, cerebellum and the lateral columns of the lumbar enlargement of the spinal cord showed occasional neuronal damage varying from a loss of Nissl substance in milder cases to complete disappearance of the entire neuron in severe damage. The lateral motor column of the lumbar segment was the seat of major neuronal involvement. The most widespread and consistent change was loss of Nissl substance and shrinkage of the neuron (figs. 6 and 7). Glial clumps were all that remained of the neuron in some areas of the lateral lumbar column. Patchy axon degeneration and demyelination was observed in the sciatic nerve probably corresponding to the loss of neurons in the lateral column (figs. 8 and 9).

Liver. Liver cells of birds fed the arginine deficient diet appeared shrunken and detached (figs. 10 and 11). This was most marked toward the center of the lobule. In some areas of the mid-zone the sinusoidal endothelium had been separated from the liver cells, but overt necrosis was not observed. The portal triad and central veins appeared normal. Histochemical stains for DNA and RNA revealed no significant difference between the deficient and control groups. Hypertrophy of the nucleoli was not consistently observed.

Lymphoid tissue. The thymus and bursa of Fabricius were small and atrophic. The cortical and medullary regions were well demarcated. Cortical cells of the bursa of Fabricius were thin, loosely arranged and there was a tendency toward lumen formation in the medulla.

Feather. The feathers of the arginine-deficient chick did not develop normally. They had a frizzled appearance which was most pronounced in the feathers on the dorsal surface of the wing. The shaft was curved and the secondary components of the feathers were poorly developed. They were brittle and many were broken at the skin surface. Microscopically, the feather follicle showed varying degrees of loss of epithelium and sclerosis. Cellular infiltration about the root of the feather was frequently observed.

DISCUSSION

The results of this investigation as well as those of previous work (Hegsted et al., '41; Fisher et al., '56) seem to rule out the possibility that low muscle creatine is the primary cause of the muscle paralysis commonly observed in the arginine deficient chick. On the other hand, it is clear that arginine-deficient chicks have a lower than normal concentration of muscle creatine.

The most likely cause of ataxia and muscle attenuation is the nerve cell damage observed primarily in the spinal cord. No doubt the loss of motor neurons and the demyelination of axons in the peripheral nerves would affect the locomotor activity. Muscle atrophy would logically follow the loss of motor neurons which innervate the muscles concerned. In this regard it is significant that the leg reflexes are controlled by the area of spinal cord most severely affected.

Daniel et al. ('47) described a bone deformity which occurred in about 20% of chicks fed purified diets. The legs of the chicks splayed out and the tibiae were twisted in the same manner as observed in the arginine-deficient chicks described here. Although the deformity was attributed to an unknown factor at the time, it seems likely now that it was the result of an arginine deficieny. Twisting of the tibia probably results from a normal stress which in this case is applied to a weakened bone. Since arginine is an important constituent of collagen and other connective tissue protein, the bone matrix does not develop normally when there is a deficit of this amino acid.

It is not obvious why this study did not confirm the microscopic changes in the liver cord cells reported by Jungherr et al. ('58). The nuclear structure in the livers of the arginine-deficient chicks observed in this work exhibited some variations from normal, but the deviations were no greater than those observed in other nutritional deficiencies concerned with proteins. Differences in the experimental conditions used in the two laboratories may have influenced the histological picture. For one thing, Jungherr et al. used a basal diet that supplied 22% of casein and about 0.8% of arginine, whereas our basal diet contained 35% of casein and approximately 1.2% of arginine.

The involution and other alterations observed in the thymus and bursa of Fabricius in the arginine-deficient chick are not specific inasmuch as they have been reported in a number of conditions of stress (Newberne and McEuen, '57; Garren and Barber, '55).

SUMMARY

The ataxia and muscle paralysis commonly observed in the arginine-deficient chick were not related directly to low content of muscle creatine. Deficient chicks showed extensive motor neuron damage in the spinal cord, demyelination of peripheral nerves and atrophy of leg muscles. Abnormal bone development with a degenerate bony matrix was associated with the grossly abnormal tibia which was twisted from its normal axis. Nerve damage appeared to be sufficiently severe to explain the stilted gait of arginine-deficient chicks and the abnormal bone development accounts for the peculiar stance. Damage to the epithelium of the feather follicle probably accounts for the incomplete development of this appendage.

LITERATURE CITED

Almquist, H. J., E. Mecchi and F. H. Kratzer 1941 Creatine formation in the chick. J. Biol. Chem., *141:* 365.

Armed Forces Institute of Pathology 1957 Manual of Histologic and Special Staining Technics. Armed Forces Institute of Pathology, Washington, D.C., p. 128, 131.

Daniel, L. J., G. F. Combs, L. C. Norris and G. F. Heuser 1947 A new bone deformity in the chick. Proc. Soc. Exp. Biol. Med., *65:* 273.

Fisher, H., R. C. Salander and M. W. Taylor 1956 Growth and creatine biosynthesis in the chick as affected by the amino acid deficiences of casein. J. Nutrition, *58:* 459.

Garren, H. W., and C. W. Barber 1955 Endocrine and lymphatic gland changes occurring in young chickens with fowl typhoid. Poultry Sci., *34:* 1250.

Hegsted, D. M., G. M. Briggs, C. A. Elvehjem and E. B. Hart 1941 The role of arginine and glycine in chick nutrition. J. Biol. Chem., *140:* 191.

Jungherr, E. L., J. M. Snyder and H. M. Scott 1958 Cytopathologic changes in liver cord cells of arginine deficient chicks. J. Nutrition, *65:* 281.

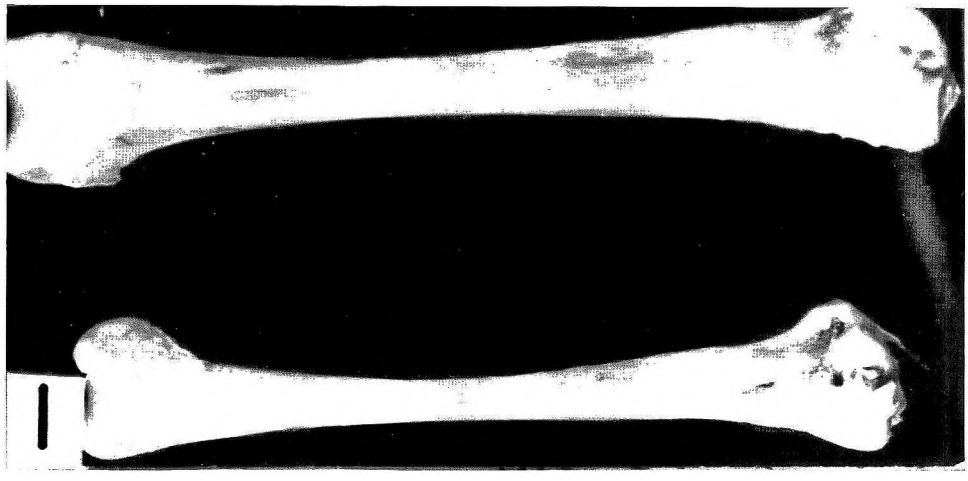
Newberne, P. M., and G. L. McEuen 1957 The influence of various levels of nitrofurazone on growth and development of chicks. Poultry Sci., *36:* 739.

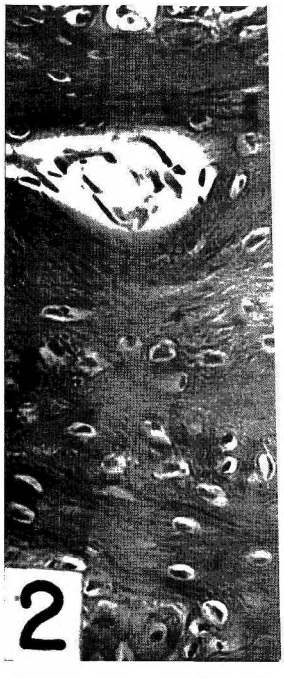
Pearse, A. G. E. 1953 Histochemistry, Theoretical and Applied. Little, Brown and Company, Boston, p. 425, 427.

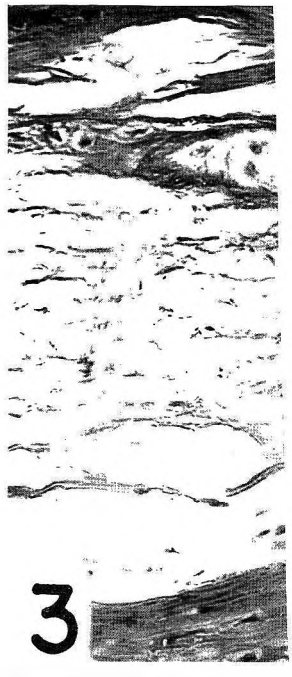
Richardson, L. R., and A. G. Hogan 1946 Diet of mother and hydrocephalus in infant rats. J. Nutrition, *32:* 459.

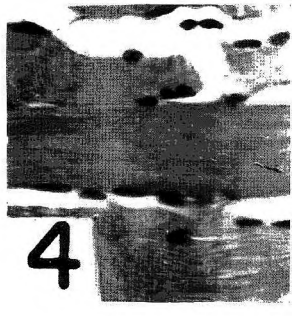
Rose, W. C., O. M. Helmer and A. Chanutin 1927 A modified method for the estimation of total creatinine in small amounts of tissue. J. Biol. Chem., *75:* 543.

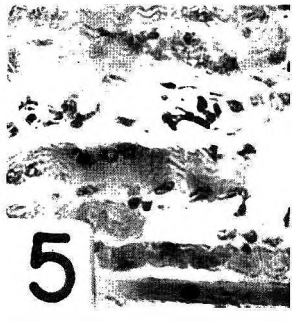
Savage, J. E., and B. L. O'Dell 1960 Arginine requirement of the chick and the arginine sparing value of related compounds. J. Nutrition, *70:* 129.











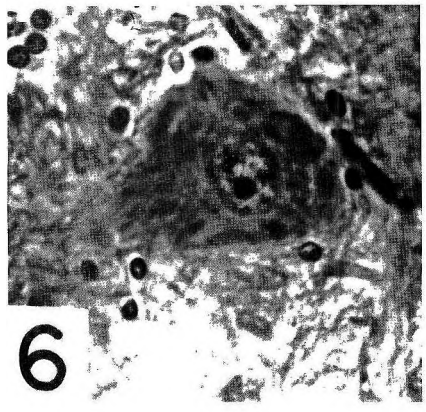
1 Gross picture of a tibia from control chick (upper) that received a diet containing 2.4% of arginine compared with a tibia from arginine deficient chick (lower) that received 1.2%. Note flattening of the bony shaft in the distal third of the deficient bone and the lateral rotation of the condyle.

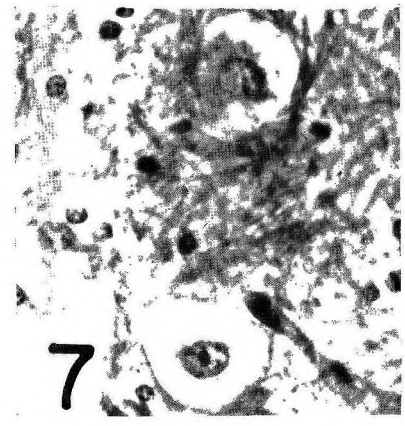
2 Longitudinal section of tibia from control chick (2.4% of arginine). Hematoxylin and eosin. × 400.

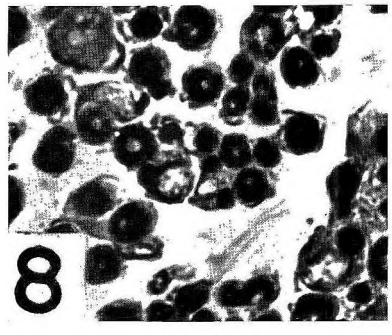
3 Longitudinal section of a tibia from an arginine deficient chick (1.2% of arginine). Only a degenerate, fibrillary structure remains. It contains scattered pyknotic nuclei of osteocytes and no ground substance. Hematoxylin and eosin. × 400.

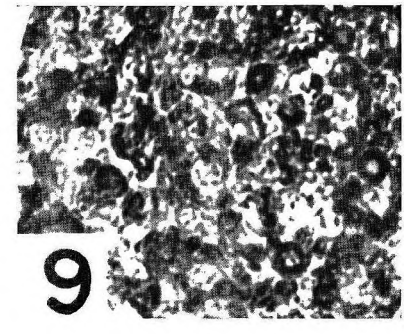
4 Longitudinal section of skeletal muscle from tibia of control chick (2.4% of arginine). Hematoxylin and eosin. × 440.

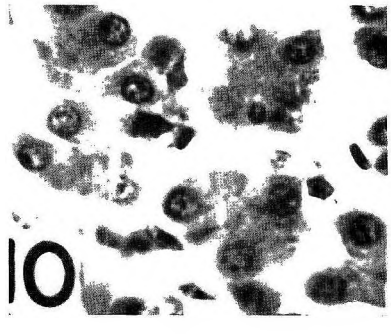
5 Section comparable to figure 4 from arginine-deficient chick (1.8% of arginine). The muscle fibers are thin and have lost many of their striations. In the center a fiber has disappeared leaving only the connective tissue stroma with a relative increase in nuclei. Hematoxylin and eosin. × 440.

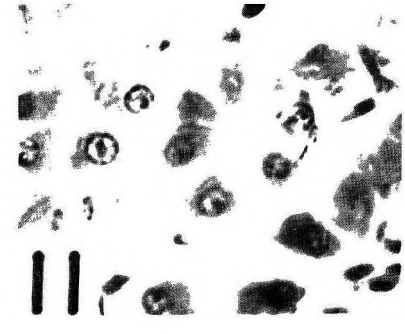












6 Motoneuron from the lateral column of spinal cord of a control chick (2.4% of arginine). The nucleus, nucleolus and chromatin are pronounced and cytoplasmic Nissl substance abundant. Gallocyanin. × 1680.

7 Motoneurons from an area comparable with figure 6 but from a deficient chick (1.2% of arginine). There is degeneration and shrinkage of the nucleus and loss of cytoplasmic Nissl substance. Gallocyanin. × 1680.

8 Sciatic nerve from control chick. The myelinated fibers stain deeply. The separation of fibers is an artifact due to sectioning. Sudan black B. × 800.

9 Sciatic nerve from arginine-deficient chick (1.2% of arginine). Some fibers show little or no myelin while others appear normal. Sudan black B. × 800.

10 Liver from control chick (2.4% of arginine). Hematoxylin and eosin. × 435.

11 Liver from arginine-deficient chick (1.2% of arginine). Cells are shrunken and detached from each other and from the sinus endothelium. Hematoxylin and eosin. × 435.

Cellulose Metabolism in the Rat

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The extent of cellulose metabolism by animals remains poorly defined in certain instances, although in some cases, namely, ruminants and Equidae, there is extensive utilization of cellulose. It has been shown that certain bacteria are able to degrade cellulose[1] (Hungate, '50), and the bacterial degradation of cellulose is utilized by the ruminant to convert cellulose into digestible compounds (McClymont, '51). Similarly, it has been suggested that animals which possess a cecum achieve considerable digestion of cellulose by virtue of the cecal micropopulation (Dukes, '42; Maynard and Loosli, '56). Digestion of cellulose by the nonruminant may be accomplished in a similar manner depending upon the cellulose degradative ability of the gastrointestinal (GI) micropopulation, the passage time and the internal conditions of the GI tract. The extent of cellulose metabolism in the rat, a nonruminant, has been investigated and is presented in this report. An isotope technique was employed to determine the utilization of cellulose.

PROCEDURE

Tobacco cellulose-C^{14}, purified by the method of Wise et al. ('39), was provided for this investigation by the Medical College of Virginia, Richmond, Virginia.[2] Soybean cellulose-C^{14} was isolated from the ground pods, leaves, stems and roots of plants grown at the Argonne National Laboratory. The soybean cellulose was purified by treatment with benzene, 10% NaOH, 10% KOH and further purified with sodium chlorite (Jayme, '42) to remove lignin.

A gas train was used to assay the Carbon-14 content of a rat's expired air after feeding cellulose-C^{14} (fig. 1). Commercial rat

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[1] Sijpesteijn, A. K. 1948 Cellulose-decomposing bacteria from the rumen of cattle. Doctoral thesis, University of Leiden, Leiden, Holland.

[2] The authors wish to acknowledge the cooperation and generosity of Dr. P. S. Larson in providing the tobacco cellulose-C^{14} for this study.

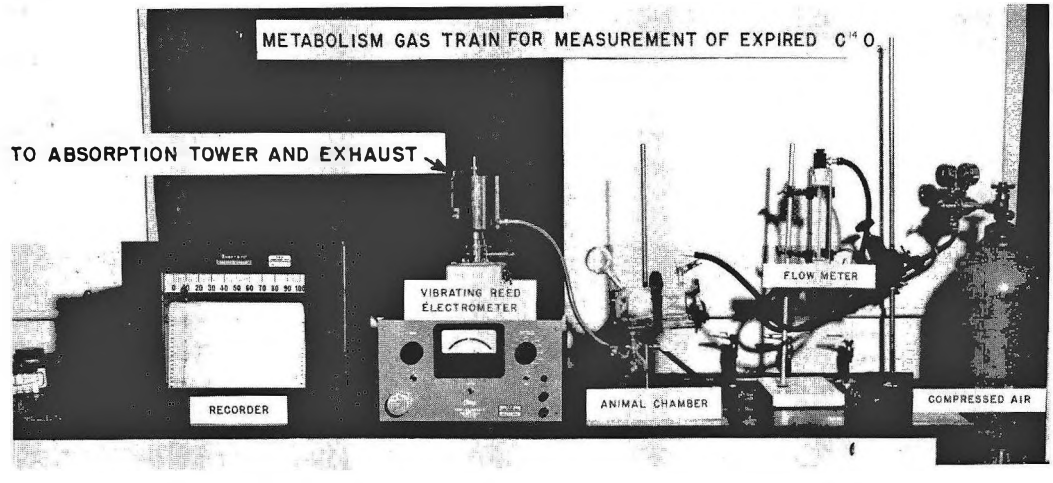

Fig. 1 Metabolism gas train for measurement of expired $C^{14}O_2$.

chow[3] was available in the food cup. The gas train included a tank of compressed air, regulating valves and a flow meter, an air-tight metabolism chamber designed to retain urine and feces,[4] a one-liter ionization chamber connected to a Cary vibrating reed electrometer and a Minneapolis-Honeywell recorder. An air flow rate of 500 cm^3 per minute sustained the rat in the metabolism chamber and the effluent gases were exhausted out the window after passing through a soda-lime absorption tower.

Thirty milligrams of tobacco cellulose-C^{14} (1.13 μc/mg) or 100 mg of soybean cellulose-C^{14} (9.03×10^{-2} μc/mg) were force-fed with a hypodermic syringe fitted with a nasopharyngeal cannula. Immediately after feeding, the rat was placed in the metabolism chamber and the C^{14} content of the rat's expired air was assayed until the expired air again contained no detectable $C^{14}O_2$ (72 to 96 hours). The area under the strip chart trace was then determined with a polar planimeter and converted to curies of C^{14} with the formula

$$\frac{v \times \text{min.}}{R} \times \frac{1}{\text{Calib. const.}} \times \frac{\text{vol./min.}}{\text{vol.}} = \text{curies}$$

R = resistance in ohms
v = volts
min. = minutes
vol. = chamber volume in liters
Calibration constant = amperes/curie

The C^{14} content of the urine, feces and carcass was also determined with the ionization chamber-vibrating reed electrometer system. In this procedure, the ionization chamber of the vibrating reed electrometer was evacuated, and dried samples of urine or water homogenates of the feces or carcass were drawn into the ionization chamber with air as they were combusted with the Van Slyke-Folch reagent ('40). The recorder measured the C^{14} content of each combusted sample as a signal (volts) on the strip chart, and the signals were subsequently converted to curies of radioactivity. The Carbon 14 retained in the body tissues was determined when $C^{14}O_2$ was no longer detectable in the expired air. The animals used in this study were 250 to 350-gm male rats supplied by the Sprague-Dawley Company, Madison, Wisconsin.

RESULTS AND DISCUSSION

The data show that the rat metabolizes 31% of ingested cellulose. Twenty-four per cent of the fed radioactivity is excreted by way of the expired air. A much smaller amount, 1% of the fed radioactivity, is excreted in the urine and 6% is retained in the carcass (table 1). The ability to metabolize cellulose varies somewhat as shown by the standard deviation of the results, although most of the data cluster closely about the mean figures. Variation in the ability to digest cellulose could not be ascribed to any gross pathological state and no intensive health study was made since all of the rats appeared normal and healthy.

The fecal C^{14} (58% of the fed activity) was higher than the C^{14} content of the expired air, indicating the most of the cellulose was passed through the rat.[5] It is not known whether unaltered cellulose

[3] Purina Rat Chow, Ralston Purina Company, St. Louis, Missouri.

[4] Collection of feces was facilitated by two floor screens in the metabolism chamber which were supported on points pushed from the glass walls. The lower screen, 8 mesh, caught the feces and held them away from the urine. The upper screen, 2 mesh, supported the rat and made coprophagy difficult by its separation (¾ inch) from the lower screen.

[5] Coprophagy, observed only occasionally, was detected by a second rise in the $C^{14}O_2$ expiration late in the experimental period. Experimental results were discarded when coprophagy was observed.

TABLE 1

Metabolism of cellulose-C^{14} by the normal rat

Cellulose	No. of animals	C^{14} activity in terminal site			
		Breath	Feces	Urine	Carcass
		%	%	%	%
Tobacco	15	23.5 ± 5.1[1]	57.6 ± 12.1	0.8 ± 0.5	5.6 ± 5.2
Soybean	3	24.9 ± 2.5	58.1 ± 4.6	1.0 ± 0.6	6.2 ± 5.6

[1] Standard deviation.

was excreted since the chemistry of the excreted cellulose was not investigated. The fecal C^{14} may represent insoluble precursors of the metabolizable catabolites (Gray and Pilgrim, '52) or unaltered cellulose, depending upon the passage time and the cellulose degradative activity of the micropopulation. Conrad et al., ('58) reported the presence of petroleum ether extractable C^{14} in rat feces after feeding soybean cellulose-C^{14}. A very small amount of radioactivity, approximately 1% of the amount fed, was excreted in the urine. The chemical nature of the urine radioactivity has not been established, but it may possibly be a precursor of the expired radioactivity. The results also show that the distribution of radioactivity into the expired air, urine and feces when soybean cellulose-C^{14} was fed was almost identical to the distribution observed when tobacco cellulose-C^{14} was fed. It appears that the soybean cellulose-C^{14} and the tobacco cellulose-C^{14} are utilized equally well by the rat.

The role of the rat gastrointestinal micropopulation. The role of the rat GI micropopulation in the metabolism of cellulose was also investigated. It is known that the ruminant is able to metabolize cellulose by virtue of its rumen micropopulation, (Elsden and Phillipson, '48; Huffman, '53), but the importance of the GI micropopulation to the nonruminant has not been clearly defined. The GI micropopulation of the rat is antibiotic-sensitive and it was found that oxytetracycline (12.5 mg intraperitoneally for three days) reduced the coliform and anaerobic bacterial colony count from $7 \times 10^6/cm^2$ to $2.2 \times 10^2/cm^2$.[6] A marked reduction in the ability to metabolize cellulose was observed after treatment with oxytetracycline, while oral sulfasuxidine given alone or in conjunction with penicillin and streptomycin (intraperitoneally) was found less effective in lowering the expiration of $C^{14}O_2$. It was concluded that the lethality of oxytetracycline for the GI micropopulation was more pronounced than that of sulfasuxidine or sulfasuxidine with penicillin plus streptomycin since oxytetracycline caused the greatest reduction in metabolism of cellulose, but reduced the fecal retention time no more than the other antibiotics which also caused diarrhea. The oxytetracycline-treated rats excreted zero and 2.5% of the fed radioactivity in the expired air while 99 and 92% of the radioactivity was excreted in the feces (table 2). The significant reduction in the ability of the oxytetracycline-treated rats to metabolize cellulose indicates that the GI micropopulation is essential for the metabolism of cellulose by the rat as is the rumen micropopulation in the case of the bovine and other species of ruminants.

Cellulose metabolism in the rat cecum. The site of cellulose metabolism in the rat GI tract was also investigated. The cecum in many animals is somewhat analogous to the bovine rumen in being a capacious enlargement of the GI tract. It is a likely location for the reactions of cellulose metabolism since the interior conditions of the cecum, that is pH, aeration and agitation, are similar to conditions in the rumen where cellulose metabolism occurs in the ruminant. Cellulose metabolism by cecectomized rats[7] was studied in order to as-

[6] Media: coliform bacterial colonies: desoxycholate agar, 46 gm/1000 ml; anaerobic bacterial colonies: H_2O, 1000 ml; nutrient broth, 8 gm; agar, 17 gm; Na thioglycollate, 2 gm; dextrose, 10 gm; Na formaldehyde sulfoxylate, 1 gm; methylene blue, 1 ml (1/500 dilution); pH 7.6 to 7.8, adjusted with NaOH. Media streaked with infusion broth preparation (coliform colonies) or feces slurry (anaerobic colonies) and incubated aerobically or anaerobically for 24 to 48 hours prior to counting.

[7] Cecectomy performed by one of the authors, Donald A. Peterson.

TABLE 2

Metabolism of tobacco cellulose-C^{14} by rats with oxytetracycline-sterilized gastrointestinal tracts

Rat no.	C^{14} activity in terminal site			
	Breath	Feces	Urine	Carcass
	%	%	%	%
1	0	99.1	0.1	—
2	2.6	91.8	0.1	—

TABLE 3

Metabolism of tobacco cellulose-C^{14} by cecectomized rats

Rat no.	C^{14} activity in terminal site			
	Breath	Feces	Urine	Carcass
	%	%	%	%
3	0	87.7	0.1	—
4	1.5	81.4	0.1	—

certain the extent of cecal metabolism. Cecectomized rats were fed tobacco cellulose-C^{14} and metabolism was followed in the previously described manner. It was observed that the cecectomized rats are virtually unable to metabolize cellulose as was noted with the oxytetracycline-treated rats. The expired air of two cecectomized rats contained zero and 1.5% of the fed radioactivity similar to the expired radioactivity observed with the oxytetracycline-treated rats (table 3). The fecal C^{14}, 87.7 and 81.4% of the fed radioactivity, was somewhat lower than the fecal C^{14} of the oxytetracycline-treated rats, indicating that cellulose metabolism also occurs outside of the cecum, probably in the colon. These results show that the cecum is the primary site of cellulose metabolism in the rat.

SUMMARY

1. Investigations with tobacco and soybean cellulose-C^{14} have shown that the rat used in these studies metabolizes 31% of ingested cellulose. Twenty-four per cent of the radioactivity is excreted in the expired air, 1% is excreted in the urine and 6% is retained in the body tissues.

2. Digestion of cellulose in these laboratory rats was markedly reduced by oxytetracycline, showing that the gastrointestinal (GI) micropopulation, which is antibiotic-sensitive, is essential for cellulose metabolism in the rat.

3. Cecectomized rats were virtually unable to digest cellulose, indicating that the cecum is the site of cellulose metabolism in the rat.

4. The significant reductions in the ability to metabolize cellulose indicate that the GI micropopulation is essential for the metabolism of cellulose by the rat as is the rumen micropopulation in the case of the bovine and other species of ruminants.

ACKNOWLEDGMENT

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LITERATURE CITED

Conrad, H. E., W. R. Watts, J. M. Iacono, H. F. Kraybill and T. E. Friedemann 1958 Digestibility of uniformly labeled Carbon-14 soybean cellulose in the rat. Science, *127:* 1293.

Dukes, H. W. 1942 The Physiology of Domestic Animals. Comstock Publishing Company, New York, p. 328.

Elsden, S. R., and A. T. Phillipson 1948 Ruminant digestion. Ann. Rev. Biochem., *17:* 705.

Gray, F. V., and A. F. Pilgrim 1952 Origins of the volatile fatty acids in the rumen. Nature, *170:* 375.

Huffman, C. F. 1953 Ruminant nutrition. Ann. Rev. Biochem., 22: 399.

Hungate, R. E. 1950 The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev., *14:* 1.

Jayme, G. 1942 Preparation of holocellulose and cellulose with sodium chlorite. Cellulose Chem., 20: 43.

Maynard, L. A., and J. K. Loosli 1956 Animal Nutrition. McGraw-Hill, New York, p. 45.

McClymont, G. L. 1951 Identification of the volatile fatty acid in the peripheral blood and rumen of cattle and the blood of other species. Australian J. Agr. Res., 2: 92.

Van Slyke, D. D., J. Folch and J. Plazin 1940 Manometric carbon determination. J. Biol. Chem., *136:* 509.

Wise, L. E., F. C. Peterson and W. M. Harlow 1939 The action of ethanolamine on woody tissue. Indust. Engineer. Chem. Anal. Ed., *11:* 18.

Role of Diet Lipids in the Appearance of Dystrophy and Creatinuria in the Vitamin E-Deficient Rat[1,2]

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There is evidence that most if not all of the signs of tocopherol deficiency in animals are related to the peroxidation of higher unsaturated lipids[3,4] (Horwitt et al., '56; Zalkin and Tappel, '60), rather than to the loss of any specific tocopherol-containing enzyme (Donaldson et al., '58). In man, tocopherol need appears to be a function of the level of intake of polyunsaturated fatty acid, or for practical purposes, linoleic acid. This requirement is very low when the intake of these fatty acids is low[5,6] (Horwitt, '60). Briggs et al. ('56) showed that encephalomalacia did not occur in chicks fed a fat-free diet, and more recently, Bieri and Briggs[7] demonstrated that chicks could be raised with full egg laying potential with a vitamin E-free diet. Crider and associates[8] have raised rats through three generations without vitamin E by substituting other antioxidants.

It has been shown that the amount of tocopherol needed by chicks to prevent encephalomalacia can be related to the intake of linoleic acid-containing lipids[9] (Century et al., '59; Century and Horwitt, '59). Cod liver oil inhibited the occurrence of encephalomalacia (Century and Horwitt, '59), although studies by other investigators have shown that fish and cod liver oils in the diet increased the incidence of encephalomalacia in tocopherol-deficient chicks (Zacharias et al., '50; Singsen et al., '55).

In the present work, studies of the relationship of tocopherol need to the lipids ingested have been extended to rats. The tocopherol requirement of rats fed a 15% corn oil diet was evaluated by determining the level of supplementation necessary to permit optimal weight gain, and to prevent increased creatine excretion, as well as higher concentrations and turnover of nucleic acids in skeletal muscle. An attempt was also made to reverse creatinuria in rats which had been fed for 71 weeks a 15% corn oil diet without vitamin E. Finally, animals were given tocopherol-deficient diets containing cod liver oil, linseed oil, corn oil, olive oil or coconut oil, and the effects of different lipids on the appearance of creatinuria, dystrophy and other signs of vitamin E deficiency were observed.

EXPERIMENTAL

Tocopherol requirement of rats fed 15% of corn oil. Weanling Sprague-Dawley

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[1] The support of Illinois Mental Health Fund, The National Vitamin Foundation, Inc., and U.S.P.H.S. grant no. A-1126 is gratefully acknowledged.

[2] A preliminary report of this work was given at the 1960 meeting of the Federation of American Societies for Experimental Biology, Chicago: Century, B., and M. K. Horwitt 1960 Metabolic changes in tocopherol deficient rats. Federation Proc., *19:* 222 (abstract).

[3] Bieri, J. G., and G. M. Briggs 1959 Nonessentiality of vitamin E for normal growth and development of the chick. Federation Proc., *18:* 517 (abstract).

[4] Trucco, R. E., M. P. Carpenter, P. B. McCay, A. E. Kitabchi and R. Caputto 1960 Malonaldehyde formation and inhibition of gulonolactone oxidase in vitamin E deficiency. Federation Proc., *19:* 419 (abstract).

[5] Horwitt, M. K., C. C. Harvey and B. J. Meyer 1958 Plasma tocopherol, hemolysis, and dietary unsaturated lipid relationships in man. Federation Proc., *17:* 245 (abstract).

[6] Horwitt, M. K. 1959 Tocopherol requirements of man. Federation Proc., *18:* 530 (abstract).

[7] See footnote 3.

[8] Crider, Q. E., P. Alaupovic and B. C. Johnson 1960 Vitamin E and antioxidants in the nutrition of the rat. Federation Proc., *19:* 420 (abstract).

[9] Century, B., and M. K. Horwitt 1958 Some factors in production of encephalomalacia in the vitamin E deficient chick. Federation Proc., *17:* 473 (abstract).

TABLE 1

Basal diets with isocalorically substituted lipids

Ingredient	15% fat	7% fat	0.2% fat
	gm	*gm*	*gm*
Casein (vitamin-test)	25	25	25
Salts (USP 14)	4	4	4
Dextrose	30	39	48
Cornstarch	26	33.2	42
Coconut oil, olive oil or corn oil	15	—	—
Linseed oil, or cod liver oil	—	7.8	—
Low fat (as corn oil)	—	—	0.24
Choline dihydrogen citrate	0.33	0.33	0.33
Vitamin mix[1]	0.33	0.33	0.33

[1] Vitamin mix contains (in milligrams) *i*-inositol, 11.1; *p*-aminobenzoic acid, 11.1; D-calcium pantothenate, 6.2; 2-methyl-1-4-naphthoquinone, 5.0; niacin, 10.0; thiamine·HCl, 2.2; pyridoxine·HCl, 2.2; riboflavin, 2.2; folic acid, 0.2; and biotin, 45 μg; vitamin B_{12}, 3 μg; and starch to 0.33 gm.

male rats were assigned a 15% corn oil diet (table 1) and given zero, 0.2, 0.4, 0.7 or 1.0 mg of *d*-α-tocopheryl acetate per week orally. Tocopherols were almost completely eliminated from the corn oil by vacuum distillation[10] and by stirring in air at 95°C for about two hours until a peroxide number of 50 was reached. Corn oil, choline dihydrogen citrate and water-soluble vitamins were mixed into the diet daily to minimize external lipid peroxidation. Vitamins A (2500 IU) and D (350 IU) were given weekly. Creatine and creatinine excretions in 24-hour urine collections were determined by the method of Bonsnes and Taussky ('45). At intervals up to 53 weeks, rats were given 10 μc of sodium formate-C^{14} intraperitoneally in saline containing 100 μc per ml, and sacrificed after one, two or 4 hours. Liver, kidney, skeletal muscle and brain were frozen until analyzed. Nucleic acids were extracted essentially by the method of Schneider ('45), with modifications by Davidson and Smellie ('52). Cold 5-ml aliquots of 20% aqueous homogenates of liver and 25% homogenates of kidney, muscle, and brain were mixed with 10 ml of cold 10% trichloroacetic acid (TCA) and centrifuged. The precipitate was suspended and successively washed with 10 ml of cold TCA, 12 ml of 80% ethanol, 10 ml of 95% ethanol, and three 10-ml washings of 2:1 ethanol-ether heated to boiling in a water bath. Nucleic acids were extracted from the residue by heating three times with 6-ml portions of 10% NaCl in a boiling water bath for one-half hour. The extracts were pooled, diluted to 20 ml, and duplicate 1-ml aliquots were evaporated to dryness, digested with perchloric acid, and analyzed for total phosphorus (Zilversmit and Davis, '50). Nucleic acids in 15 ml of NaCl extract were precipitated overnight with 30 ml of cold ethanol and centrifuged at 2000 rpm for 20 minutes. The supernatant was thoroughly drained, and the precipitate was dissolved in warm water and diluted to 10 ml. Duplicate 2-ml aliquots were dried on lightly oxidized copper planchettes and C^{14} determinations made with a flow-counter, with corrections for self-absorption. Duplicate 1-ml aliquots of liver and kidney nucleic acids and 2-ml aliquots from muscle and brain were analyzed for phosphorus. Specific activities were determined as counts per minute per micromole of phosphorus (cpm/μmP).

Effects of various diet lipids on tocopherol-deficiency in the rat. In order to study the effect of varying the amounts of linoleic acid and higher unsaturated fatty acids in the diet, weanling male rats were given the tocopherol-deficient diets shown in table 1, containing (in per cent) coconut oil, 15; olive oil, 15; corn oil, 15; linseed oil, 7; cod liver oil, 7; or corn oil (low fat) 0.2 respectively. The low level of corn oil in the last diet was intended to be just sufficient to supply the essential fatty

[10] Stripped corn oil was prepared at our request by Distillation Products Industries, Rochester, New York, for a study of human requirements of tocopherol in progress for over 6 years, and also for animal studies.

TABLE 2
Composition of diet lipids

Lipid	Peroxide no.	Iodine no.	Diene	Triene
			%	%
Coconut oil	2	9	1.2	—
Olive oil	23	86	13.6	0.6
Corn oil	54	129	52	0.9
Linseed oil	120	182	11.8	54
Cod liver oil[1]	45	160	2	1.6

[1] Also tetraene, 3.2%; pentaene, 12%; and hexaene, 14%.

acid requirement. Lipids were substituted isocalorically with the carbohydrates. For each diet, 4 rats were given 4 mg of *d*-α-tocopheryl acetate per week, and 6 were unsupplemented. Olive oil (USP), cod liver oil (USP), raw linseed oil (NF) and coconut oil were obtained commercially without added antioxidants and aerated at 95°C until tocopherols were virtually destroyed. Peroxide numbers of the lipids treated in this manner are indicated in table 2. Destruction of tocopherols by this method has minimal effects on the compositions of the diet lipids, with no observable physiological effect in rats (Andrews et al., '60), and in chicks, changes in the peroxide number (PN) did not influence the incidence of encephalomalacia, once the tocopherol was destroyed (Century et al., '59). Iodine number (Hanus), PN, and polyunsaturated fatty acids (American Oil Chemists' Society, '57) of the diet lipids were determined and results are shown in table 2. After 32 weeks, the rats were sacrificed, examined grossly, and samples of skeletal muscle taken for histological examination.

RESULTS

Tocopherol requirement of rats fed 15% corn oil. The need for tocopherol in rats fed a 15% corn oil diet with a highly unsaturated fatty acid composition (table 2) is demonstrated in tables 3 and 4 and in figure 1. The average weight curves in

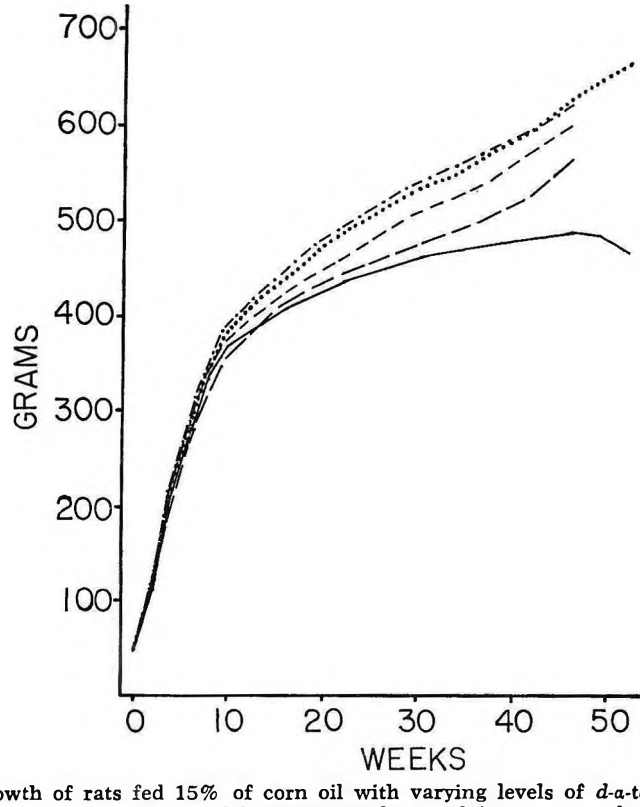

Fig. 1 Growth of rats fed 15% of corn oil with varying levels of *d*-α-tocopheryl acetate supplementation. —, none; — —, 0.2 mg per week; - - -, 0.4 mg per week; - . - ., 0.7 mg per week; . . . . . , 1.0 mg per week.

figure 1 were corrected for small discontinuities due to the periodic sacrifice of rats. The numbers of animals initially given supplements of zero, 0.2, 0.4, 0.7 and 1.0 mg of *d*-α-tocopheryl acetate per week were 18, 8, 8, 8, and 17, respectively; and by 46 weeks, the numbers remaining were 10, 6, 5, 6 and 10, respectively. Growth was significantly depressed in unsupplemented rats ($P < 0.001$), and slightly lowered in animals given only 0.2 mg of vitamin E per week ($P < 0.05$). Gross dystrophy was evident in the unsupplemented animals, and severe atrophy and cellular infiltrations of skeletal muscle were seen histologically by 53 weeks.

High creatine excretion was observed in unsupplemented rats by 19 weeks (table 3). After 28 weeks, creatinuria was significantly elevated in rats supplemented with 0.2 mg of vitamin E per week, and by 40 weeks, in animals given as much as 0.4 mg per week. Normal daily creatine excretion averaged from 13 to 17 mg/kg in rapidly growing rats, but declined to about 6 mg/kg when the animals were 43 weeks old. At this time, even rats given 0.4 mg of vitamin E per week excreted over 15 mg/kg of creatine per day. Compensatory decreases in creatinine excretion were first observed after 28 weeks in rats given 0.0 or 0.2 mg of tocopherol per week.

Higher levels of nucleic acid phosphorus were found in skeletal muscle from unsupplemented rats after 27 to 31 weeks, and in animals given 0.2 mg of vitamin E per week, after 40 to 53 weeks (table 4). These observations are consistent with excessive nucleation reported to occur in nutritional dystrophy (Martin and Moore, '39; Mackenzie and Mackenzie, '59). A slight increase in liver nucleic acid phosphorus was also observed in unsupplemented rats after 40 weeks. Higher uptake of formate-C^{14} into skeletal muscle nucleic acids was observed in all rats given zero or 0.2 mg of vitamin E per week (table 4), and also in animals given 0.4 mg per week for 40 to 53 weeks, when they were sacrificed one hour after injection of labeled formate. No differences were seen in the uptake or formate-C^{14} by liver, kidney or brain nucleic acids from deficient rats.

Reversal of tocopherol deficiency in rats. Following tocopherol depletion for 71 weeks with a 15% corn oil diet, three groups of 4 rats each were given, zero 1.5 or 6.0 mg of *d*-α-tocopheryl acetate per week, respectively, for a period of 10 weeks. The average starting weight of these rats was about 600 gm. Rats given 1.5 mg per week gained 54 gm, and those given 6.0 mg per week gained 50 gm, while the unsupplemented animals lost 3 gm during this time.

TABLE 3

Creatine and creatinine excretion from tocopherol-deficient rats fed a 15% stripped corn oil diet

Weeks	Vitamin E	No. of rats	Creatine	Creatinine	Ratio
	mg/week		*mg/kg*	*mg/kg*	
19	0.0	18	39.4 ± 3.1[1]	24.8 ± 1.0	1.63 ± 0.12[1]
	0.2	8	21.8 ± 2.2	22.4 ± 2.3	1.00 ± 0.09
	0.4	8	16.7 ± 2.4	24.3 ± 1.9	0.69 ± 0.08
	0.7	8	13.4 ± 2.0	25.1 ± 2.4	0.60 ± 0.13
	1.0	17	16.7 ± 2.0	26.0 ± 1.6	0.71 ± 0.11
28	0.0	18	41.4 ± 2.4[1]	24.2 ± 0.8[1]	1.73 ± 0.10[1]
	0.2	8	26.4 ± 3.5[1]	26.2 ± 0.3[3]	1.02 ± 0.14[1]
	0.4	8	15.4 ± 2.7	27.5 ± 1.1	0.56 ± 0.10
	0.7	8	9.4 ± 2.7	28.2 ± 1.0	0.35 ± 0.11
	1.0	17	10.5 ± 1.7	29.1 ± 0.9	0.37 ± 0.06
40	0.0	14	35.8 ± 3.0[1]	16.9 ± 0.6[1]	2.16 ± 0.19[1]
	0.2	6	23.3 ± 4.1[1]	19.4 ± 1.5[2]	1.42 ± 0.24[1]
	0.4	5	15.8 ± 6.6[3]	21.1 ± 1.4	0.75 ± 0.31[3]
	0.7	6	5.6 ± 1.6	21.8 ± 2.0	0.26 ± 0.07
	1.0	14	6.7 ± 1.3	24.3 ± 0.9	0.26 ± 0.05

[1] $P < 0.001$.
[2] $P < 0.01$.
[3] $P < 0.05$.

TABLE 4

Nucleic acid phosphorus levels and uptake of formate-C¹⁴ into nucleic acids in tissues of tocopherol-deficient rats fed 15% stripped corn oil diet

Weeks	Vitamin E	No. of rats	Nucleic acid phosphorus			
			Muscle	Liver	Kidney	Brain
	mg/week		*mg/100 gm*	*mg/100 gm*	*mg/100 gm*	*mg/100 gm*
28 to 31	0.0	4	18.6±1.9[1]	92.2±2.5	64.0±1.2	24.2±1.6
	0.2	2	14.2±0.8	92.5±0.3	62.4±1.4	26.0±0.6
	0.4	3	13.1±1.4	93.0±1.4	66.9±1.8	25.8±1.1
	0.7	2	13.2±0.4	93.1±1.7	69.8±2.6	25.0±2.2
	1.0	3	11.8±1.4	86.8±1.4	63.4±3.2	23.4±1.4
40 to 53	0.0	13	20.7±1.0[2]	102.3±2.5[1]	63.0±1.6	29.2±1.1
	0.2	6	16.7±1.4[3]	96.8±1.4	65.2±1.3	30.5±1.8
	0.4	5	15.3±1.5	91.3±2.8	69.1±3.7	30.7±2.1
	0.7	6	14.5±1.3	91.0±3.4	63.5±1.6	31.0±1.4
	1.0	11	12.8±0.5	92.6±3.5	63.1±1.6	27.7±1.2

	Hours sacrificed			Nucleic acid C¹⁴			
				Muscle	Liver	Kidney	Brain
				cpm/μMP	*cpm/μMP*	*cpm/μMP*	*cpm/μMP*
28 to 31	4	0.0	4	142±11[3]	38±5	77±10	27±4
		0.2	2	103±10[1]	43±9	88±2	21±0.5
		0.4	3	68±1	43±2	124±17	32±4
		0.7	2	63±14	93±13	134±51	28±4
		1.0	4	59±6	60±6	113±20	30±4
40 to 53	1	0.0	4	85±6[2]	48±5	70±2	17±2
		0.2	3	57±2[3]	52±16	92±18	16±2
		0.4	2	40±3[1]	60±29	114±15	16±1
		0.7	3	31±3	56±12	71±7	16±0.5
		1.0	4	24±4	51±11	82±10	15±4
	2	0.0	4	116±9[2]	42±10	73±7	22±4
		0.2	3	101±13[3]	51±15	95±5	24±3
		0.4	3	47±3	43±3	83±3	21±3
		0.7	3	48±13	50±14	124±20	26±2
		1.0	4	44±7	67±11	104±11	30±6
	4	0.0	5	158±26[3]	45±9	99±16	26±6
		1.0	3	44±8	70±27	126±28	35±7

[1] $P < 0.05$. [2] $P < 0.001$. [3] $P < 0.01$.

Control rats, which were given 4.0 mg of vitamin E per week throughout the entire experiment, gained 32 gm from an initial weight of 832 gm. High levels of urinary creatine were slightly decreased in three out of 4 rats given 1.5 mg of vitamin E per week, and appreciably lowered in animals fed 6.0 mg per week (fig. 2). Almost all of the changes occurred during the first week, but the low levels of creatine excretion seen in control animals were not attained. Creatinuria in the unsupplemented animals continued to increase during the same period.

Effects of varying dietary lipids on tocopherol deficiency in the rat. No growth impairment was seen in tocopherol-deficient rats fed low fat or 15% of coconut oil for 32 weeks (fig. 3). At the end of the same period, weight gain was significantly depressed in tocopherol-deficient rats given 7% of cod liver oil ($P < 0.001$), 7% of linseed oil ($P < 0.01$), and 15% of corn oil ($P < 0.05$). Rats fed 0.2% of corn oil and 15% of coconut oil ingested an estimated 15 and 30 mg of linoleic acid per day, respectively, which may or may not have been barely adequate to prevent essential fatty acid deficiency.

The severity of various signs of deficiency appeared to be related to the degree of peroxidizability of the lipids in the diet (table 5). Gross signs of muscular dystrophy were slight-to-moderate in deficient animals given 15% of olive oil, marked in rats given 15% of corn oil and most severe

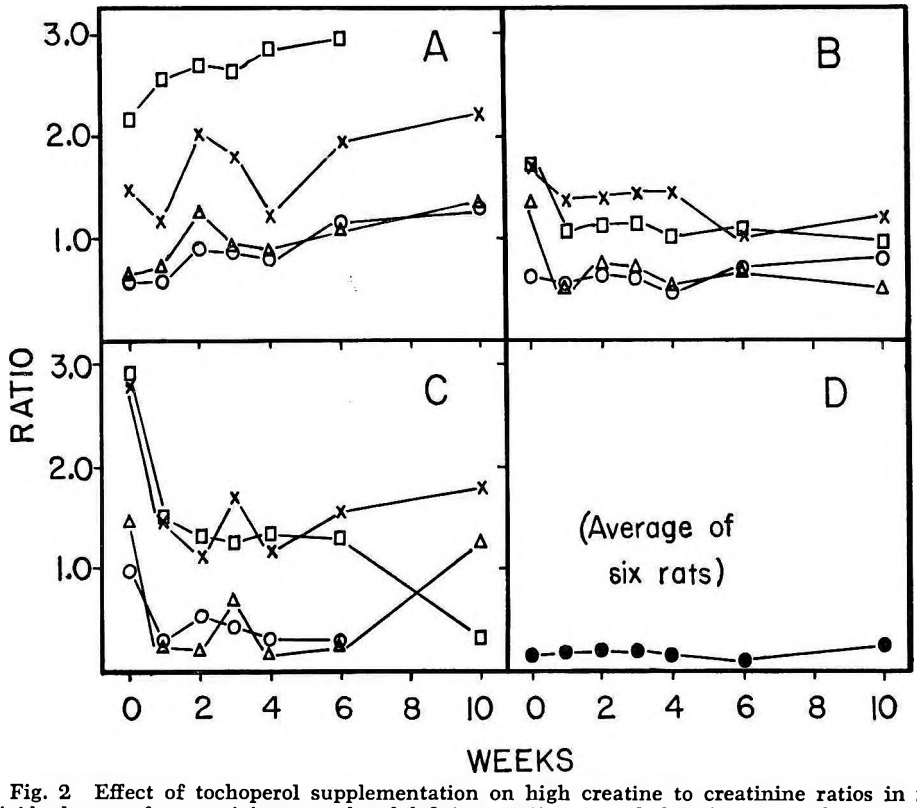

Fig. 2 Effect of tochoperol supplementation on high creatine to creatinine ratios in individual rats after receiving tocopherol-deficient 15% corn oil diet for 71 weeks. Supplements: A, none; B, 1.5 mg *d*-α-tocopheryl acetate per week; C, 6.0 mg *d*-α-tocopheryl acetate; D, control animals given 4.0 mg *d*-α-tocopheryl acetate per week throughout entire experiment.

in those fed 7% of linseed oil or cod liver oil. Animals receiving the last two diets were emaciated in appearance, and somewhat irritable but not incapacitated. Microscopic sections of skeletal muscle from tocopherol-deficient rats fed linseed oil and cod liver oil diets revealed extensive areas of focal cellular infiltrations and basophilic, degenerating fibers. Sections from deficient animals given 15% of corn oil showed somewhat fewer lesions, while muscle from rats given 15% of olive oil had few infiltrating lesions with some evidence of hyalinization and degeneration. No gross nor microscopic signs of dystrophy were seen in tocopherol-deficient rats fed 0.2% of corn oil or 15% of coconut oil, nor in any animals supplemented with tocopherol, regardless of diet. The depot fat in deficient rats fed 7% of linseed or 7% of cod liver oil was severely depleted and brown. Small hemorrhagic and erosion-type ulcers were seen in the lower stomach and duodenal mucosa, which appeared to correlate slightly with tocopherol deficiency and intake of unsaturated lipids. The occurrence of these gastrointestinal ulcers will be discussed in a separate paper in conjunction with similar findings in adult men fed unsaturated lipids.

No changes in creatine excretion were observed in vitamin E-deficient rats fed 15% of coconut oil or low fat diets, whereas by 11 weeks, elevated creatinuria was observed in tocopherol-deficient rats receiving 7% of cod liver oil, and after 15 weeks, in animals given 7% of linseed oil or 15% of corn oil (fig. 4). Deficient animals fed 15% of olive oil showed a milder increase in creatinuria, which was significant by 19 weeks.

TABLE 5
Various effects of tocopherol deficiency in rats fed various lipid diets

Diet		Gross appearance	Depot fat	Gastro-intestinal ulcers	Skeletal muscle	
					Gross	Microscopic degeneration
Corn oil, 0.2%	E−	normal	ample; white	1/6	normal	0
	E+	normal	ample; white	0/4	normal	0
Coconut oil, 15%	E−	normal	ample; white	2/6	normal	0
	E+	normal	ample; white	0/4	normal	0
Olive oil, 15%	E−	somewhat gaunt	ample; white	2/5	slight dystrophy	±
	E+	normal	ample; white	2/4	normal	0
Corn oil, 15%	E−	gaunt; tremors	depleted; yellow	4/5	moderate to severe atrophy and fibrosis	+++
	E+	normal	ample; white	1/4	normal	0
Linseed oil, 7%	E−	very gaunt; tremors	brown; depleted	6/6	severe atrophy and fibrosis	+++
	E+	normal	ample; white	3/4	normal	0
Cod liver oil, 7%	E−	very gaunt; tremors and weakness	brown; depleted	3/6	severe atrophy and fibrosis	+++
	E+	normal	ample; white	0/4	normal	0

DISCUSSION

The data presented suggest that tocopherol requirement in the rat is dependent upon the amount and degree of peroxidizability of the lipid ingested. Cod liver oil and linseed oil, which provided highest levels of the more unsaturated fatty acids, were most effective in producing dystrophy, elevated creatine excretion, and brown fat in tocopherol-deficient animals. These animals were gaunt and had little depot fat. The effects of 15% of corn oil appeared to be equivalent to those of 7% of linseed oil, except that the former diet produced little discoloration of depot fat. Only mild dystrophy and creatinuria were observed in vitamin E-deficient rats given 15% of olive oil and none of these signs were seen in animals given 15% of coconut oil or 0.2% of corn oil. Observations of "brown fat" in tocopherol-deficient rats receiving highly unsaturated lipid diets were similar to those of Filer and associates ('46), who compared the effects of fatty acid esters of cod liver oil, linseed oil and corn oil.

Evans and Emerson ('43) found that 0.6 mg per week of *d*-α-tocopheryl acetate maintained normal growth and muscle striation in rats receiving a diet containing 22% of lard and 2% of cod liver oil, but up to 4 mg per week was needed to prevent sterility in males over a period up to 9 months. Unfortunately, the amounts of linoleic acid in the lard used are not known, so it is difficult to make comparisons with the 15% corn oil diet used in the study (table 2). The data reported here indicate that about 0.7 mg of *d*-α-tocopherol per week was sufficient over a one-year period to permit normal weight gain, and to prevent increased creatine excretion, elevated nucleic acid phosphorus in skeletal muscle, and higher uptake of formate-C^{14} into muscle nucleic acids of rats fed a diet containing 25% of casein and 15% of corn oil. The increased turnover of muscle nucleic acids in tocopherol-deficient rats was similar to that observed by Dinning ('55). The latter investigator did not observe impairment of weight gain nor increased nucleic acid phosphorus in muscle, but this may have been due to the shorter experimental period of 5 months and less unsaturated fat in the diet.

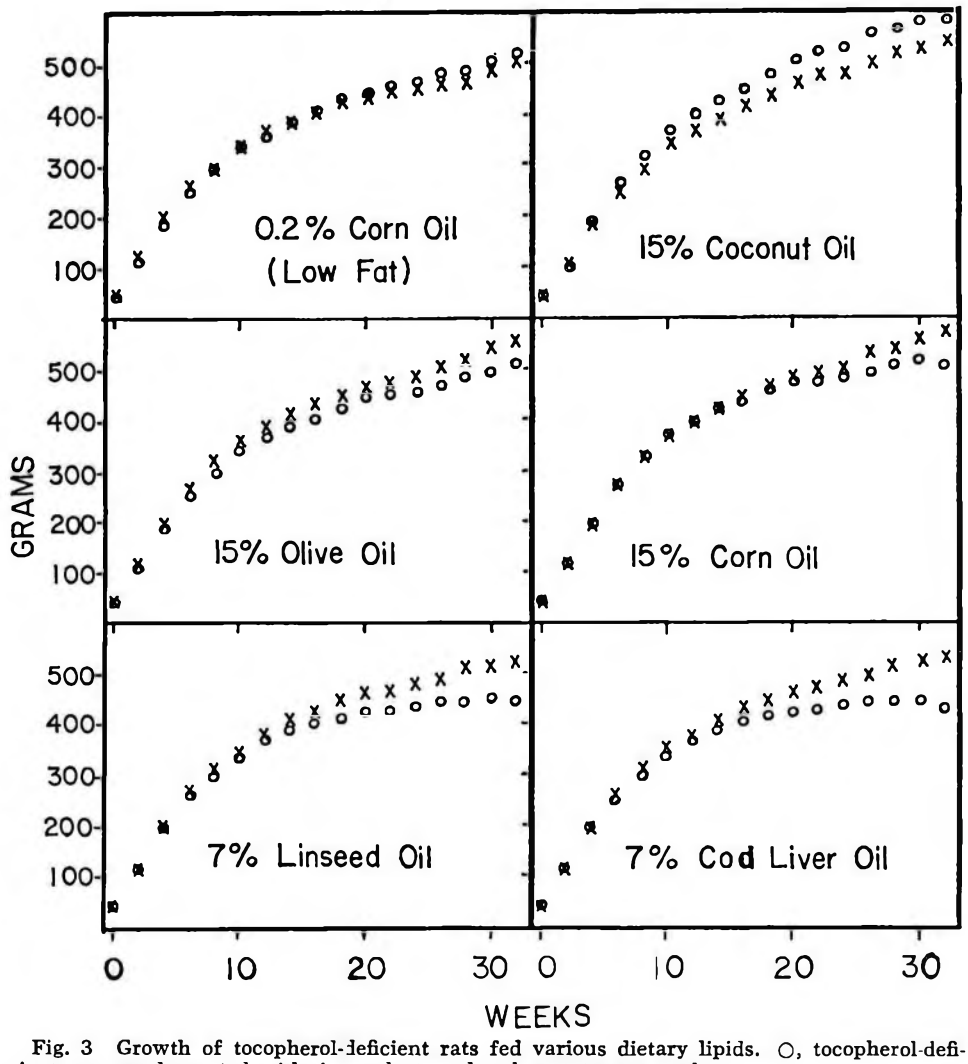

Fig. 3 Growth of tocopherol-deficient rats fed various dietary lipids. ○, tocopherol-deficient; ×, supplemented with 4 mg *d*-α-tocopheryl acetate per week.

Creatinuria observed in rats fed a vitamin E-deficient diet containing 15% of corn oil for 71 weeks could only be partly reversed with 6.0 mg of *d*-α-tocopheryl acetate per week, although excellent weight gain was observed in deficient animals given 1.5 and 6.0 mg per week. Early reports suggested that tocopherol resupplementation does not cure nutritional dystrophy in rats (Martin and Moore, '39; Mackenzie et al., '40), but Mackenzie and Mackenzie ('59) have shown more recently that elevated creatinuria in vitamin E-deficient rats could be reversed within 6 days and muscular lesions reversed within 14 days by giving 5 mg of *dl*-α-tocopheryl acetate twice weekly to rats made dystrophic with a tocopherol-deficient diet for 23 weeks. It is possible that feeding a highly unsaturated vitamin E-deficient diet for 71 weeks induced a greater degree of irreversibility in our animals than that seen by Mackenzie and Mackenzie, who fed their animals 10% lard diet for 23 weeks. In this connection, it is important to point out that since the linoleic content of lard can vary from 2 to over 20%, depending upon what was

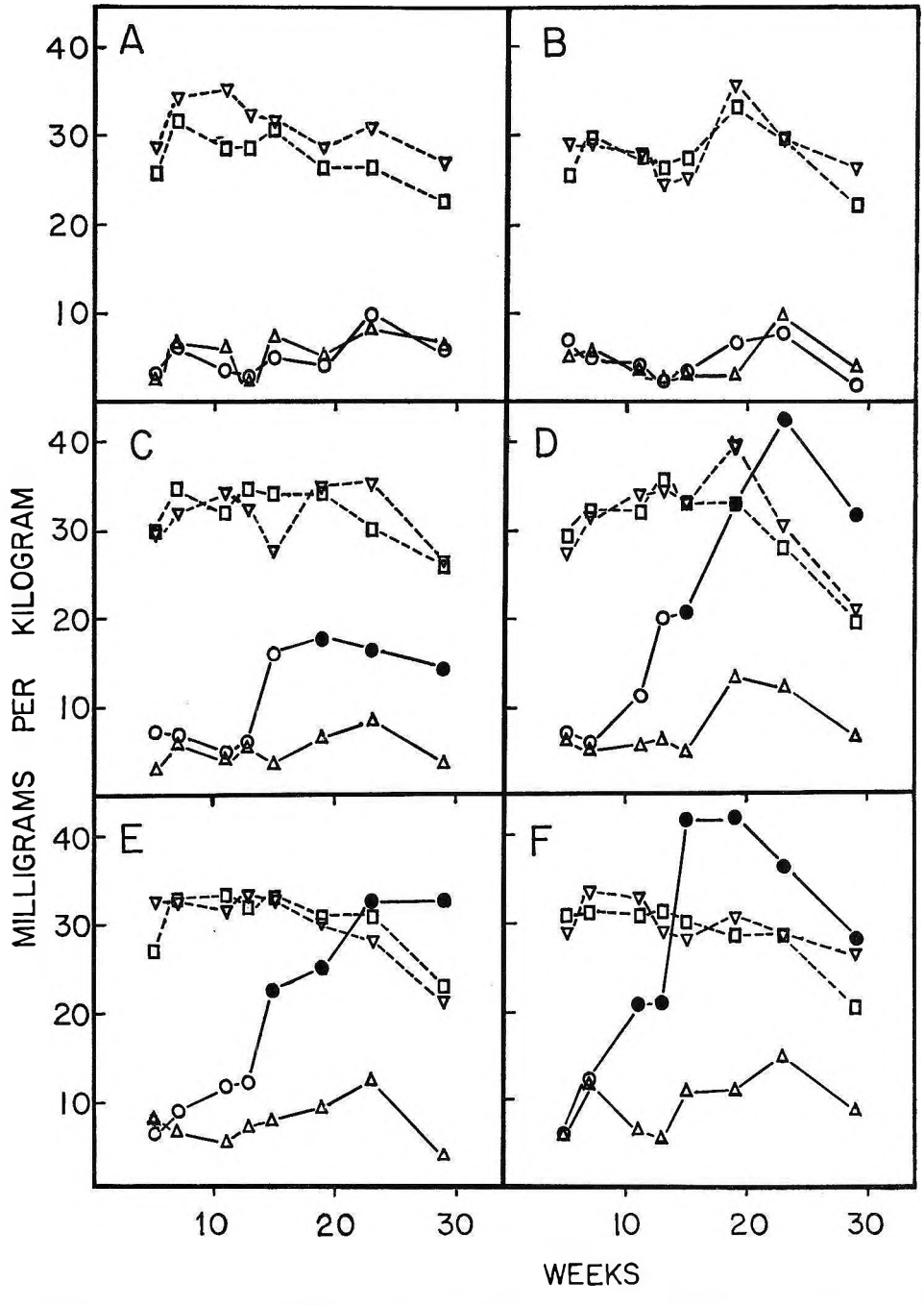

Fig. 4 Comparisons of creatine and creatinine levels in urine of tocopherol-deficient and tocopherol-supplemented rats fed various dietary lipids. Diets: A, 0.2% corn oil (low fat); B, 15% coconut oil; C, 15% olive oil; D, 15% corn oil; E, 7% linseed oil; F, 7% cod liver oil. ○—○, urine creatine of unsupplemented rats (filled circles represent statistically significant points); △—△, urine creatine of vitamin E-supplemented rats; □- - -□, urine creatinine of unsupplemented rats; ▽- - -▽, urine creatinine of vitamin E-supplemented rats.

fed to the pig, it becomes essential in all future studies to designate the fatty acid composition of the lard used (Horwitt, '60).

Moore and co-workers ('56) showed that degeneration of the testes and *in vitro* hemolysis of erythrocytes with dialuric acid occurred in tocopherol-deficient rats fed fat-free diets. This may be explained by noting that the animals are started on the experiment with a store of peroxidizable lipids which may be preferentially retained by the tissues (Horwitt et al., '59), and that the hemolysis test is a function of the ratio of tocopherol to peroxidizable lipid (Horwitt, '60). Thus, the oxidation of unsaturated lipids in erythrocytes even from rats receiving a fat-free diet may be expected to be accelerated by dialuric acid *in vitro* in the absence of tocopherol. Similarly, high levels of polyunsaturated fatty acids are found in the testes in rats receiving a low intake of oxidizable lipids (Aaes-Jorgensen, '58). These are capable of being peroxidized in the absence of sufficient protective levels of tocopherol. How much of these unsaturated fatty acids arises from pre-experimental stores in the animal and how much is synthesized *in vitro* remains to be determined.

SUMMARY

In rats fed unsaturated fatty acids to the extent found in 15% of corn oil, supplements of 0.4 mg of *d*-α-tocopherol per week permitted normal growth and prevented increased nucleic acid phosphorus levels in skeletal muscle, while 0.7 mg per week prevented increased creatinuria and higher labeling of muscle nucleic acids with formate-C^{14}.

Renewed weight gain was observed in rats supplemented with 1.5 and 6.0 mg per week following a period of 71 weeks of feeding a 15% corn oil tocopherol-deficient diet, but only partial reversal of the creatinuria was seen in animals given as much as 6.0 mg per week.

Muscular dystrophy and increased creatine excretion were produced to a high degree of tocopherol-deficient rats fed 7% cod liver oil, 7% linseed oil, and 15% corn oil diets, to a lesser degree in those given 15% of olive oil, and not at all in animals fed 15% of coconut oil or 0.2% of corn oil (low fat). After 32 weeks, severe depletion and considerable brown discoloration of depot fat were seen in vitamin E-deficient rats fed the cod liver oil and linseed oil diets. When 15% of corn oil was fed, the depot fat was depleted but only mildly discolored. No changes were seen in the adipose tissue of rats given 15% of coconut oil or 0.2% of corn oil.

Tocopherol requirement appeared to be related to the amount of peroxidizable fatty acids ingested and/or stored in the tissues.

ACKNOWLEDGMENTS

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LITERATURE CITED

Aaes-Jorgensen, E. 1958 Essential fatty acid deficiency. III. Effects of conjugated isomers of dienoic and trienoic fatty acids in rats. J. Nutrition, *66:* 465.

American Oil Chemists' Society 1957 Official and Tentative Methods, Cd. 7–48, Poly-unsaturated fatty acids. Chicago.

——— 1957 Official and Tentative Methods, Cd. 8–53, Peroxide values, Chicago.

Andrews, J. S., W. M. Griffith, J. F. Mead and R. A. Stein 1960 Toxicity of air-oxidized soy bean oil. J. Nutrition, *70:* 199.

Bonsnes, R. W., and H. H. Taussky 1945 On the colorimetric determination of creatinine by the Jaffe reaction. J. Biol. Chem., *158:* 581.

Briggs, G. M., M. R. S. Fox and J. G. Bieri 1956 Growth of chicks without dietary vitamin E. Poult. Sci., *35:* 1134.

Century, B., and M. K. Horwitt 1959 Effects of fatty acids on chick encephalomalacia. Proc. Soc. Exp. Biol. Med., *102:* 375.

Century, B., M. K. Horwitt and P. Bailey 1959 Lipid factors in the production on encephalomalacia in the chick. Arch. Gen Psychiat., *1:* 420.

Davidson, J. N., and R. M. S. Smellie 1952 Phosphorus compounds in the cell. 2. The separation by ionophoresis on paper of the constituent nucleotides of ribonucleic acids. Biochem. J., *52:* 594.

Dinning, J. S. 1955 The role of vitamin E in regulating the turnover rate of nucleic acids. J. Biol. Chem., *212:* 735.

Donaldson, K. O., A. Nason and R. H. Garrett 1958 The role of lipids in electron transport. IV. Tocopherol as a specific cofactor of mammalian cytochrome C reductase. Ibid., *233:* 572.

Evans, H. M., and G. A. Emerson 1943 The prophylactic requirement of the rat for alpha tocopherol. J. Nutrition, *26:* 555.

Filer, Jr., L. J., R. E. Rumery and K. E. Mason 1946 Specific unsaturated fatty acids in the production of acid-fast pigment in the vitamin E-deficient rat and the protective action of tocopherols. First Conference on Biological Antioxidants, Josiah Macy, Jr., Foundation, p. 67.

Horwitt, M. K. 1960 Vitamin E and lipid metabolism in man. Am. J. Clin. Nutrition, *8:* 451.

Horwitt, M. K., C. C. Harvey and B. Century 1959 Effect of dietary fats on fatty acid composition of human erythrocytes and chick cerebella. Science, *130:* 917.

Horwitt, M. K., C. C. Harvey, G. D. Duncan and W. C. Wilson 1956 Effects of limited tocopherol intake in man with relationships to erythrocyte hemolysis and lipid oxidations. Am. J. Clin. Nutrition, *4:* 408.

Mackenzie, J. B., and C. G. Mackenzie 1959 The effect of α-tocopherol, α-tocopherylhydroquinone and their esters on experimental muscular dystrophy in the rat. J. Nutrition, *67:* 223.

Mackenzie, C. G., J. B. Mackenzie and E. V. McCollum 1940 Occurrence of tremors and incoördination in vitamin E-deficient adult rats. Proc. Soc. Exp. Biol. Med., *44:* 95.

Martin, A. J. P., and T. Moore 1939 Some effects of prolonged vitamin E deficiency in the rat. J. Hygiene, *39:* 643.

Moore, T., I. M. Sharman and R. J. Ward 1956 The effect of dietary fat on various signs of vitamin E deficiency in the rat. Biochem. J., *64:* 13P.

Schneider, W. C. 1945 Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., *161:* 293.

Singsen, E. P., L. M. Potter, L. D. Matterson, R. H. Bunnell, A. Kozeff and E. L. Jungherr 1955 Studies on encephalomalacia in the chick. 4. The influence of oil in fish meal, oils from various species of fish, and animal fats on the incidence of encephalomalacia. Poult. Sci., *34:* 1075.

Zacharias, L., P. Goldhaber and V. E. Kinsey 1950 Vitamin E-deficiency in chicks. I. The effects of dietary supplements on plasma tocopherol levels and vitamin E-deficient symptoms. J. Nutrition, *42:* 359.

Zalkin, H., and A. L. Tappel 1960 Studies of the mechanism of vitamin E action. IV. Lipide peroxidation in the vitamin E-deficient rabbit. Arch. Biochem. Biophys., *88:* 113.

Zilversmit, D. B., and A. K. Davis 1950 Microdetermination of plasma phospholipids by trichloracetic acid precipitation. J. Lab. Clin. Med., *35:* 155.

Antibiotics and Plasma Cholesterol in the Mouse

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A considerable body of evidence has accumulated to support the conclusion that the bacterial flora of the gut exerts a major effect upon bile acid production and plasma cholesterol concentration and, therefore, possibly upon the development of atheroma. Lindstedt and Norman ('56) found the biological half-life of $-C^{14}$ labeled cholic acid to be 2 to 3 days in normal rats but that this half-life increased to 10 to 15 days in animals fed a diet containing a mixture of oxytetracycline and phthalylsulfathiazole. Later Gustafsson et al. ('57) reported the biological half-life of cholic acid to be 8 to 14.5 days in germ-free rats. When these same animals were transferred to nonsterile areas the cholic acid half-life dropped to that observed previously with normal animals. Further, Danielsson and Gustafsson ('59) have shown that germ-free rats have higher concentrations of plasma cholesterol than normal controls. Forbes and associates ('58) on the other hand, found no differences in serum cholesterol of chicks reared under germ-free conditions and those maintained under normal conditions.

Portman and associates ('56) have observed that the feeding of succinylsulfathiazole to rats receiving a sucrose diet has no effect on serum cholesterol; however, addition of this drug to a starch-containing diet resulted in elevation of serum cholesterol values to the level of that in the rats fed sucrose. In a like manner, Kritchevsky et al. ('58) have reported that chlortetracycline raises the serum cholesterol levels of chicks fed a diet containing glucose and cholesterol but is without effect when administered with a diet containing sucrose.

Nelson and associates ('53) and Goldberg and Smith ('54) have studied the effect of chlortetracycline on cholesterol concentration in the blood of the rabbit. The former observed that addition of chlortetracycline to a commercial ration containing cholesterol produced increases in estimated vascular damage, serum cholesterol and total serum lipids. The latter used two diets, one without fat and a second containing 12.5% of cottonseed oil. In the low-fat groups chlortetracycline was initially observed to produce lowered levels of lipids which later increased and exceeded those found in control animals. In the groups receiving cottonseed oil, chlortetracycline was found to lower both free and esterified cholesterol concentrations in the plasma and tissues.

Encrantz and Sjövall ('59) have recently investigated the production of bile acids in man as a function of age. Only conjugated bile acids were found in the duodenal contents of infants and no desoxycholic acid or conjugates thereof were present. These differences may well be due to differences in microflora of the intestine. Norman and Sjövall ('58, '59) have demonstrated the conversion of cholic acid to several products including desoxycholic acid in the intestine of the rat. These substances appear to have been formed by bacteria since they are not found in the feces of animals reared under germ-free conditions. It has also been demonstrated that the hydrolysis of bile acid conjugates takes place only in the rat intestine. In addition, both this hydrolysis and the conversion of cholic to desoxycholic acid have been effected *in vitro* by bacteria isolated from rat feces (Encrantz and Sjövall, '59; Norman and Grubb, '55).

It is the purpose of this communication to record some of the observations made in a study of the effect of antibiotics on plasma cholesterol concentrations in the mouse. These findings take on added significance in light of the recent report of Samuel and Steiner ('59) that neomycin

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reduces the plasma cholesterol concentration in man.

EXPERIMENTAL PROCEDURE

The weanling male mouse was selected for the experimental animal in these studies for a variety of reasons which included: (1) small space required for housing; (2) low food consumption facilitating preparation of diets and allowing use of small amounts of critical materials in short supply; (3) rapid metabolic rate making possible short experiments; (4) low cost; and (5) constant supply of excellent animals from the Merck Sharp and Dohme colony. Further it was felt that, during rapid growth, the young animal might be less able to compensate for dietary stresses and yield information not forthcoming from adults.

The basal diet contained 20% of casein,[1] 4% of salt mixture (Hegsted et al., '41), 2% of cellulose[2] and 74% of glucose.[3] A vitamin addendum supplied in milligrams for each 100 gm of diet: vitamin A palmitate, 0.4; calciferol, 0.005; 2-methylnaphthoquinone, 1.0; thiamine·HCl, 0.8; riboflavin, 1.6; pyridoxine·HCl, 0.8; niacinamide, 4.0; Ca pantothenate, 4.4; *p*-aminobenzoic acid, 4.0; choline methionine tartrate, 600; inositol, 20; folic acid, 0.2; biotin, 0.02; and vitamin B_{12}, 0.03. All supplements were added at the expense of glucose.

The mice were fed the basal diet for approximately 36 hours after weaning and animals weighing 9.5 to 11.5 gm were then allocated to the experimental groups, 8 per group. They were housed in individual wire-bottom cages and given food and water ad libitum. They were weighed at intervals, and at the end of the experimental period, usually 12 days, were decapitated under chloroform anaesthesia to obtain pooled blood samples. Total plasma cholesterol was determined by the method of Abell et al. ('52) which uses the Liebermann-Burchard color reaction.

RESULTS

The mixture of oxytetracycline and succinylsulfathiazole, the latter in place of the phthalylsulfathiazole used by Lindstedt and Norman ('56) in the rat, was incorporated into the purified diet described above.[4] A supplement of a highly saturated hydrogenated coconut oil,[5] was also used since earlier experiments had shown that it accentuated the hypercholesterolemic effect of cholesterol and cholic acid. It seemed reasonable to predict that this mixture, if it prolonged the action of cholic acid, whether of exogenous or endogenous source, would elevate the plasma cholesterol of the experimental animals receiving

[1] Labco, The Borden Co., New York.
[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[3] Cerelose, Corn Products Refining Company, Argo, Illinois.
[4] The authors are grateful to the Lederle Laboratories for their generous gifts of chlortetracycline and to Chas. Pfizer and Company, Inc., for a liberal supply of oxytetracycline.
[5] Hydrol, Durkee Famous Foods, Chicago.

TABLE 1

Effect of antibacterial substances on plasma cholesterol levels of mice receiving predominantly saturated fat

Dietary supplement	Plasma cholesterol[1]	
	Exp. 1	Exp. 2
	mg/100 ml	*mg/100 ml*
1 10% Hydrogenated coconut oil,[2] 1% cholesterol, 0.1% cholic acid	245	232
2 Supplement 1 + 0.375% oxytetracycline + 2% succinylsulfathiazole	398	268
3 10% Hydrogenated coconut oil,[2] 1% cholesterol	149	136
4 Supplement 3 + 0.375% oxytetracycline + 2% succinylsulfathiazole	203	196

[1] Each individual value in this and subsequent tables was obtained by analysis of pooled blood from a group of 8 mice.
[2] Hydrol.

TABLE 2

Comparison of effect of oxytetracycline (OT) and chlortetracycline (CT) on plasma cholesterol of the mouse under diverse dietary conditions

Supplement	Plasma cholesterol			Difference OT — CT	P
	Control	OT[1]	CT[1]		
	mg/100 ml	*mg/100 ml*	*mg/100 ml*		
None	93.5 ± 2.0[2](4)[3]	101.5 ± 3.6(4)			
	99.2 ± 2.2 (9)		81.1 ± 2.6(9)	20.4	< 0.01
10% Hydrogenated coconut oil[4]	130.0 ± 5.9 (4)	136.0 ± 5.8(4)			
	128.3 ± 3.7 (6)		130.2 ± 4.4(6)	5.8	> 0.1
2% Cod liver oil	79.2 ± 2.0 (5)	74.8 ± 4.1(8)			
	80.1 ± 1.5 (18)		54.1 ± 1.8(20)	20.7	< 0.01
10% Hydrogenated coconut oil,[4] 1% cholesterol	139.8 ± 9.0 (4)	184.2 ± 12.0(4)			
	139.4 ± 5.9 (5)		157.3 ± 15.2(5)	26.9	> 0.1
2% Cod liver oil, 1% cholesterol	106.4 ± 5.0 (5)	91.2 ± 7.0(4)			
	106.2 ± 4.2 (6)		75.0 ± 3.0(5)	16.2	0.07
10% Hydrogenated coconut oil,[4] 1% cholesterol, 0.1% cholic acid	263.2 ± 6.1 (5)	315.4 ± 10.2(8)			
	263.2 ± 6.1 (5)		223.1 ± 16.4(7)	92.3	< 0.01
2% Cod liver oil, 1% cholesterol, 0.1% cholic acid	161.0 ± 9.7 (4)	148.0 ± 3.6(5)			
	161.0 ± 9.7 (4)		120.2 ± 9.0(4)	27.8	0.02

[1] Antibiotics used at 0.2% of diet.
[2] Mean ± standard error.
[3] Number of groups of 8 mice each shown in parentheses.
[4] Hydrol.

cholic acid and cholesterol. This indeed proved to be the case as shown in table 1. In these experiments a marked elevation of plasma cholesterol resulted from the inclusion of the antibiotic mixture. In the light of the variation observed in subsequent experiments, as shown in table 2, this elevation was undoubtedly significant.

When oxytetracycline and succinylsulfathiazole were fed separately to mice receiving the cholic acid-containing diet, both were found to elevate plasma cholesterol. Unexpectedly, however, chlorotetracycline exerted a hypocholesterolemic effect (table 3) which also was significant. In some range-finding experiments a maximum response was obtainable with as little as 0.2% of either antibiotic.

A comparison of the two tetracyclines under a variety of dietary conditions yielded the results shown in table 2. Neither antibiotic produced an effect when the hydrogenated coconut oil or that and cholesterol were the dietary supplements; but in all other cases the effects of the two compounds were quantitatively different.

A number of other antibiotics were tested under the dietary conditions shown in table 2. In limited testing tetracycline acted very much the same as the hydroxy derivative. Neomycin, while yielding variable results, showed a trend under most conditions toward the elevation of plasma cholesterol. Novobiocin and chloromycetin at high levels (0.4%) at times produced a marked hypocholesterolemia and this effect was also invariably accompanied by a severe growth inhibition. Penicillin, streptomycin, bacitracin, kanamycin and sulfathiazole showed no noteworthy effects.

A cod liver oil concentrate (approximately 67% hexaenoic acid and 33% pentaenoic acid) has been shown to be a potent hypocholesterolemic agent in the rat.[6] This observation has been confirmed in the mouse and in addition a mixture of this concentrate with chlortetracycline has proved to be more effective than either alone. Interestingly enough, in the presence of corn oil and linoleic acid, which have received the most acclaim as cholesterol depressants, chlortetracycline was relatively ineffective. It produced, however, a marked hypocholesterolemic effect in the presence of corn oil and lard when small amounts of cod liver oil concentrate were also added (table 4).

[6] Personal communication from J. W. Huff.

TABLE 3

Comparison of effect of oxytetracycline, chlortetracycline and succinylsulfathiaziole on plasma cholesterol of mice receiving hydrogenated coconut oil and cholic acid

Dietary supplement	Plasma cholesterol	12-day weight gain
	mg/100 ml	*gm*
10% Hydrogenated coconut oil,[1] 1% cholesterol, 0.1% cholic acid	254	9.8
10% Hydrogenated coconut oil, 1% cholesterol, 0.1% cholic acid + 2% succinylsulfathiazole	308	1.5
10% Hydrogenated coconut oil, 1% cholesterol, 0.1% cholic acid + 0.2% oxytetracycline	312	7.0
10% Hydrogenated coconut oil, 1% cholesterol, 0.1% cholic acid + 0.375% oxytetracycline	338	5.4
10% Hydrogenated coconut oil, 1% cholesterol, 0.1% cholic acid + 0.2% chlortetracycline	173	4.6
10% Hydrogenated coconut oil, 1% cholesterol, 0.1% cholic acid + 2% succinylsulfathiazole + 0.2% oxytetracycline	324	5.2
10% Hydrogenated coconut oil, 1% cholesterol, 0.1% cholic acid + 2% succinylsulfathiazole + 0.375% oxytetracycline	384	4.5
10% Hydrogenated coconut oil, 1% cholesterol, 0.1% cholic acid + 2% succinylsulfathiazole + 0.2% chlortetracycline	231	3.0

[1] Hydrol.

TABLE 4

Effect of chlortetracycline on plasma cholesterol of mice in the presence of mixed fats

Dietary supplement	Plasma cholesterol	12-day weight gain
	mg/100 ml	*gm*
None	95	9.5
2% Corn oil	98	10.9
2% Corn oil + 0.2% chlortetracycline	97	10.6
5% Corn oil	109	10.5
5% Corn oil + 0.2% chlortetracycline	96	12.1
10% Corn oil	109	13.1
10% Corn oil + 0.2% chlortetracycline	104	11.5
1% Linoleic acid	111	11.3
1% Linoleic acid + 0.2% chlortetracycline	99	11.4
5% Linoleic acid	118	11.5
5% Linoleic acid + 0.2% chlortetracycline	95	11.8
None	112	9.7
0.2% Chlortetracycline	83	6.8
0.1% Cod liver oil concentrate	91	10.0
0.1% Cod liver oil concentrate + 0.2% chlortetracycline	54	7.5
0.5% Cod liver oil concentrate	79	10.6
0.5% Cod liver oil concentrate + 0.2% chlortetracycline	41	10.4
1.0% Cod liver oil concentrate	57	11.0
1.0% Cod liver oil concentrate + 0.2% chlortetracycline	41	11.9
10% Lard, 5% corn oil	112	12.2
10% Lard, 5% Corn oil + 0.2% chlortetracycline	118	12.0
10% Lard, 5% corn oil + 0.1% cod liver oil concentrate	107	11.4
10% Lard, 5% corn oil + 0.1% cod liver oil concentrate + 0.2% chlortetracycline	77	7.9
10% Lard, 5% corn oil + 0.5% cod liver oil concentrate	75	11.1
10% Lard, 5% corn oil + 0.5% cod liver oil concentrate + 0.2% chlortetracycline	57	9.3
10% Lard, 5% corn oil + 1.0% cod liver concentrate	70	11.3
10% Lard, 5% corn oil + 1.0% cod liver oil concentrate + 0.2% chlortetracycline	45	9.8

Several of the treatments used caused marked inhibition of growth. That this inhibition alone was not responsible for the observed changes in plasma cholesterol was shown by two experiments: (1) the food intake of mice fed the basal diet supplemented with mixed fat was restricted to such an extent that their average weight gain was only 4.5 gm in 12 days; and (2) mice were maintained ad libitum with this same diet to which was added 0.1% of quinacrine hydrochloride.[7] In a 12-day period these animals gained no weight. In neither case was there an appreciable change in plasma cholesterol.

DISCUSSION

Since it has been shown repeatedly that the oral or parenteral adminsration of cholic acid will elevate plasma cholesterol in a variety of animal species, it is not unexpected that a mixture of antibiotics which increases the biological half-life of cholic acid should also raise plasma cholesterol. What is difficult to evaluate is the failure of oxytetracycline to exert an effect in the presence of cod liver oil. The diet used by Lindstedt and Norman ('56) contained arachis oil which has no unsaturated fatty acids containing more than two double bonds. It would be interesting to know whether either or both of the antibacterial substances used by these investigators would affect the half-life of cholic acid in animals receiving penta- and hexaenoic acids.

The divergent effects of the tetracyclines stimulate speculation. Tennent et al. ('59) and Bergen et al. ('59) have demonstrated that unabsorbed basic substances may lower plasma cholesterol in animals and man, presumably by combining with and causing the excretion of bile acids. It is tempting to postulate that chlortetracycline

[7] Atabrin.

in our experiments, and neomycin in those of Samuel and Steiner ('59) are acting in a similar manner since both are basic substances. If such should be the case, however, it still must be explained why oxytetracycline does not act in the same way and why neomycin is ineffective in the mouse.

Neomycin under certain conditions has been reported to produce a malabsorption syndrome (Jacobson, '59). Chlortetracycline, too, has been shown to inhibit the absorption of an amino acid, histidine (Agar and Parker, '58). It is in the realm of possibility that both compounds are exerting their hypocholesterolemic effect by preventing the absorption or reabsorption of cholesterol or cholic acid. It may be argued that such is a more likely possibility than complexing of bile acids since absorption of most metabolites is enzyme-controlled and therefore subject to greater specificity of inhibitors. Granting that such a mechanism of action is possible, it remains to be explained why chlortetracycline exerts a hypocholesterolemic effect in the absence of highly unsaturated fats under certain dietary conditions; namely, highly saturated hydrogenated coconut oil plus cholesterol plus cholic acid, but none in the presence of the saturated fat alone, corn oil, corn oil plus lard or linoleic acid.

SUMMARY

1. Oxytetracycline and succinylsulfathiazole either alone or in combination cause an elevation of plasma cholesterol of weanling mice fed a diet containing a saturated fat, cholesterol and cholic acid. When cod liver oil, a polyunsaturated fat, was substituted for the saturated fat, oxytetracycline was without effect.

2. Chlortetracycline under identical conditions depressed plasma cholesterol.

3. When a highly unsaturated fat or mixture of highly unsaturated fatty acids was included in a cholesterol-cholic acid-free diet, chlortetracycline produced a marked hypocholesterolemic effect. In the presence of less highly unsaturated fats or fatty acids such as corn oil, corn oil plus lard, or linoleic acid, chlortetracycline exerted no effect on plasma cholesterol.

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LITERATURE CITED

Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

Agar, W. J., and M. A. Parker 1958 The inhibition of the intestinal absorption of an amino acid by chlortetracycline. Biochim. Biophys. Acta, *30:* 243.

Bergen, S. S., Jr., T. B. Van Itallie, D. M. Tennent and W. H. Sebrell 1959 Effect of an anion exchange resin on serum cholesterol in man. Proc. Soc. Exp. Biol. Med., *102:* 676.

Danielsson, H., and B. Gustafsson 1959 On serum-cholesterol levels and neutral fecal sterols in germ-free rats. Bile acids and steroids 59. Arch. Biochem. Biophys., *83:* 482.

Encrantz, J., and J. Sjövall 1959 On the bile acids in duodenal contents of infants and children. Bile acids and steroids 72. Clinica Chim. Acta, *4:* 793.

Forbes, M., R. M. Guttmacher, R. R. Kolman and D. Kritchevsky 1958 Serum cholesterol levels in germ-free chickens. Experientia, *14:* 441.

Goldberg, L., and J. P. Smith 1954 The influence of aureomycin on the response of the rabbit to cholesterol feeding. J. Path. Bact., *68:* 475.

Gustafsson, B., S. Bergström, S. Lindstedt and A. Norman 1957 Turnover and nature of fecal bile acids in germfree and infected rats fed cholic acid-24-^{14}C. Bile acids and steroids 41. Proc. Soc. Exp. Biol. Med., *94:* 467.

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

Jacobson, E. D., R. B. Chodos and W. W. Faloon 1959 An experimental malabsorption syndrome by neomycin. Clin. Res., *7:* 33.

Kritchevsky, D., W. C. Grant, M. J. Fahrenbach, B. A. Riccardi and R. F. J. McCandless 1958 Effect of dietary carbohydrate on the metabolism of cholesterol-4-C^{14} in chickens. Arch. Biochem. Biophys., *75:* 142.

Lindstedt, S., and A. Norman 1956 The excretion of bile acids in rats treated with chemotherapeutics. Acta Physiol. Scan., *38:* 129.

Nelson, D., A. C. Ivy, R. Altschul and R. Willheim 1953 Effect of aureomycin on experimental arteriosclerosis and serum cholesterol levels. Arch. Path., *56:* 262.

Norman, A., and R. Grubb 1955 Hydrolysis of conjugated bile acids by *Clostridia* and *Enterococci.* Acta Pathol. Microbiol. Scand., *36:* 537.

Norman, A., and J. Sjövall 1958 Microbial transformation products of cholic acid in the rat. Bile acids and steroids 67. Biochim. Biophys. Acta, *29:* 467.

——— 1959 On the transformation and enterohepatic circulation of cholic acid in the rat. Bile acids and steroids 68. J. Biol. Chem., 233: 872.

Portman, O. W., E. Y. Lawry and D. Bruno 1956 Effect of dietary carbohydrate on experimentally induced hypercholesteremia and hyperbetalipoproteinemia in rats. Proc. Soc. Exp. Biol. Med., *91:* 321.

Samuel, P., and A. Steiner 1959 Effect of neomycin on serum cholesterol level of man. Ibid., *100:* 193.

Tennent, D. M., H. Siegel, M. E. Zanetti, G. W. Kuron, W. H. Ott and F. J. Wolf 1959 Reduction of plasma cholesterol in animals with bile acid sequestrants. Circulation, *20:* 969.

Further Studies on the Effect of Chlortetracycline on Plasma Cholesterol of the Weanling Mouse

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Under certain dietary conditions it has been shown that chlortetracycline, but not tetracycline and oxytetracycline, when fed to growing mice causes a marked reduction in concentration of plasma cholesterol (Howe and Bosshardt, '60). This observation casts doubt on the possibility that chlortetracycline exerts its effect indirectly through an action on intestinal bacteria. Described herein are experiments designed to throw some light on this interesting phenomenon.

EXPERIMENTAL

Male weanling mice of the Merck Sharp and Dohme strain were randomized according to weight into groups of 8, housed individually and maintained with a purified-type diet described previously (Howe and Bosshardt, '60). To this diet was added 2% of cod liver oil as the sole source of fat. After feeding the experimental diets for 12 days the animals were weighed, anesthetized with chloroform and decapitated to obtain pooled blood samples for cholesterol determinations which were carried out by the method of Abell et al. ('52).

RESULTS AND CONCLUSIONS

Waller et al. ('52a) have demonstrated that chlortetracycline, when allowed to stand in strongly acid solution, is converted to anhydrochlortetracycline (fig. 1).

The dehydrated compound has greatly altered antibiotic properties (Goodman et al., '55). It has a high activity against actinomycetes but has lost much of its antibacterial activity. In our hands, when a 4% aqueous solution of chlortetracycline hydrochloride at pH 4.0 was brought to the boiling point and immediately cooled, approximately 98% of its activity against *Staphylococcus aureus* Smith was destroyed. Continued heating at the boiling point resulted in no further loss in activity.

Chlortetracycline and the heat-inactivated product were compared for effect on blood cholesterol by incorporation into the basal diet at graded levels. Both materials depressed the cholesterol level (table 1). In light of variations previously reported (Howe and Bosshardt, '60) the maximum depressions can be considered significant.

It has been postulated (Howe and Bosshardt, '60) that chlortetracycline may exert its hypocholesteremic effect in one of three ways: (1) indirectly by its effect on intestinal bacteria, (2) by precipitation or

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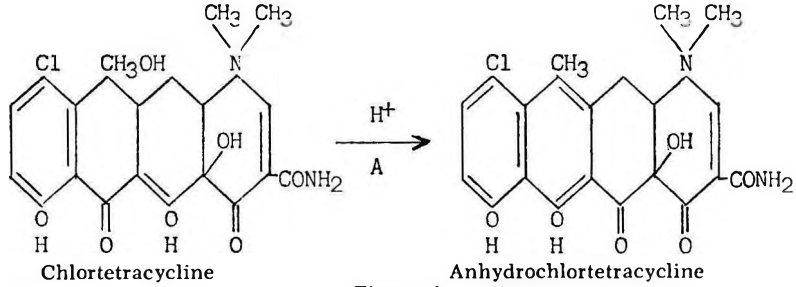

Figure 1

TABLE 1

Comparison of hypocholesterolemic effect of chlortetracycline and heat inactivated chlortetracycline in the weanling mouse

Level in diet	Chlortetracycline				Heat-inactivated chlortetracycline			
	12-Day plasma cholesterol[1]		11-Day weight gain		12-Day plasma cholesterol		11-Day weight gain	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
%	*mg/100 ml*		*gm*		*mg/100 ml*		*gm*	
0	74	75	12.3	13.7	74	75	12.3	13.7
0.05	64	56	13.2	12.6	62	57	11.1	12.2
0.1	57	51	13.0	11.4	64	44	11.9	13.3
0.2	50	31	10.1	7.7	57	47	11.3	11.9
0.4	45	31	7.4	3.7	52	51	10.3	9.1

[1] Each individual value in this and subsequent tables was obtained by analysis of pooled blood from a group of 8 mice.

complexing of bile acids or (3) by inhibition of intestinal absorption of cholesterol and bile acids. It is possible that more than one of these effects may be operating simultaneously not only with chlortetracycline, but also with the other tetracyclines. On the assumption that there may be subtle differences in the antibacterial properties of these compounds which account for the observed differences in effect on plasma cholesterol, it seemed desirable to study also the effect of heat-inactivated tetracycline hydrochloride and oxytetracycline hydrochloride on mouse plasma cholesterol. These data are shown in table 2. It is obvious that heat inactivation of either compound does not alter its effect on plasma cholesterol. It is a matter of passing interest that heating of tetracycline produced potent growth inhibitory sub-

TABLE 2

Effect of heat inactivated tetracycline and oxytetracycline on plasma cholesterol of the weanling mouse

Concentration in diet	Time of heating antibiotic	Percentage of original activity against *S. aureus* Smith	Plasma cholesterol	Weight gain
%			*mg/100 ml*	*gm*
—	—	—	84	13.1
Chlortetracycline				
0.2	0	100	50	11.9
0.4	0	100	49	8.8
Oxytetracycline				
0.2	0	100	82	11.6
0.4	0	100	85	11.5
0.2	Heated to boiling	6	81	12.4
0.4	Heated to boiling	6	84	11.1
0.2	Boiled 15 minutes	6	80	13.3
0.4	Boiled 15 minutes	6	79	12.2
0.2	Boiled 120 minutes	< 0.5	84	13.0
0.4	Boiled 120 minutes	< 0.5	86	12.6
Tetracycline				
0.2	0	100	69	12.3
0.4	0	100	66	10.8
0.2	Heated to boiling	25	75	12.7
0.4	Heated to boiling	25	68	12.7
0.2	Boiled 15 minutes	12	71	8.5
0.4	Boiled 15 minutes	12	60	2.5
0.2	Boiled 120 minutes	1	80	5.7
0.4	Boiled 120 minutes	1	63	0.1

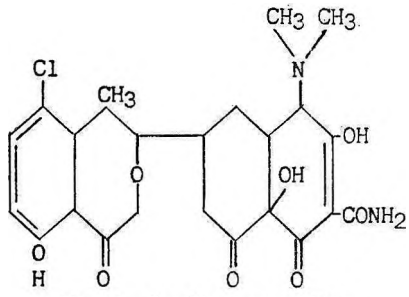

Fig. 2 Isochlortetracycline.

stances, whereas heating oxytetracycline appeared to eliminate a slight growth depressing effect.

Isochlortetracycline (Waller et al., '52b) (fig. 2), an alkaline degradation product of chlortetracycline has been reported to possess no antibacterial activity (Goldberg, '59). It seemed worthwhile to determine whether this compound, which structurally is much less closely related to chlortetracycline, but which has roughly the same molecular weight and many of the same functional groups, might also exert a hypocholesterolemic effect. The data found in table 3 show it to be completely devoid of any such activity.

Feces of normal mice have an appreciable population of *Escherichia coli*, an organism susceptible to aureomycin. To determine whether or not *E. coli* is involved in the control of the plasma cholesterol level, possibly through an effect on the reabsorption of bile secretions, a chlortetracycline resistant strain of *E. coli* was fed together with chlortetracycline. After a 10-day feeding period feces were collected from the test mice and suspensions thereof were plated on eosin methylene blue agar, a specific test for *E. coli*. As shown in table 4, chlortetracycline is equally effective in reducing the concentration of plasma cholesterol in the presence or absence of viable *E. coli* in the feces. The feces of the mice fed the chlortetracycline-resistant *E. coli* showed a heavier growth of *E. coli* than the controls fed no chlortetracycline.

The treatments used in these experiments sometimes caused inhibition of growth. Proof that growth retardation *per se* was not responsible for the observed plasma cholesterol depressions is presented elsewhere (Howe and Bosshardt, '60).

These results support the suggestion that cholortetracycline does not exert its hypocholesterolemic effect in mice indirectly through its effect on intestinal micro-organisms, nor does it seem likely that it is acting by precipitating or complexing with bile acids in the intestinal tract. Whether it acts by inhibiting ab-

TABLE 3

Comparison of hypocholesterolemic effects of chlortetracycline and isochlortetracycline

Level in diet	Chlortetracycline		Isochlortetracycline	
	12-Day plasma cholesterol	12-Day weight gain	12-Day plasma cholesterol	12-Day weight gain
%	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*
0	76	12.6	76	12.6
0.05	74	13.0	78	13.3
0.1	60	11.4	88	12.6
0.2	57	11.8	71	11.4

TABLE 4

Hypocholesterolemic effect of chlortetracycline in presence of chlortetracycline-resistant E. coli

Diet	12-Day plasma cholesterol	12-Day weight gain	Eosin methylene blue reactions
	mg/1 ml	*gm*	
2% Cod liver oil	82	13.7	+
2% Cod liver oil + 0.2% chlortetracycline	63	11.4	−
2% Cod liver oil + 0.2% chlortetracycline + chlortetracycline-resistant *E. coli*	62	11.4	+++

sorption of cholesterol or cholic acid remains to be established.

SUMMARY

1. Heating an acid solution of chlortetracycline which eliminated 98% of its activity against *Staphylococcus aureus* Smith, presumably by converting it to anhydrochlortetracycline, did not destroy its hypocholesterolemic effect in mice maintained with a cod liver oil diet. Heated acid solutions of oxytetracycline and tetracycline, which retained little of their activity against *S. aureus* Smith, still had no effect on plasma cholesterol of the mouse.

2. Isochlortetracycline incorporated into the diet of mice receiving cod liver oil did not lower plasma cholesterol of the animals.

3. Chlortetracycline depressed the plasma cholesterol concentration of mice fed a chlortetracycline-resistant strain of *Escherichia coli.*

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LITERATURE CITED

Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

Goldberg, H. S. 1959 Antibiotics, Their Chemistry and Non-Medical Uses. D. Van Nostrand Company, Princeton, p. 88.

Goodman, J. J., M. Matrishin and E. J. Backus 1955 The effect of anhydrochlortetracycline on the growth of actinomycetes. J. Bact., *69:* 70.

Howe, E. E., and D. K. Bosshardt 1960 Antibiotics and plasma cholesterol in the mouse. J. Nutrition, *72:* 368.

Waller, C. W., B. L. Hutchings, R. W. Broschard, A. A. Goldman, W. J. Stein, C. F. Wolf and J. H. Williams 1952a Degradation of aureomycin. VII. Aureomycin and anhydroaureomycin. J. Am. Chem. Soc., *74:* 4981.

Waller, C. W., B. L. Hutchings, C. F. Wolf, A. A. Goldman, R. W. Broschard and J. H. Williams 1952b Degradation of aureomycin. VI. Isoaureomycin and aureomycin. Ibid., *74:* 4981.

Effect of Bile Acids on Plasma Cholesterol in the Mouse[1]

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It has been demonstrated that elevation of the cholate content of the plasma of the rat by biliary obstruction or by continuous intravenous injection of cholate into intact rats results in a prompt hypercholesterolemia (Byers et al., '50; Friedman and Byers, '51). It has been further reported that in pathological conditions in man such as nephrosis, myocardial infarct, xanthoma and hypothyroidism which are associated with high plasma cholesterol, elevated plasma cholate is also found (Friedman et al., '52). These latter results have not been confirmed by Wysocki and associates ('55) who reported instead that in hypercholesterolemic humans and monkeys chenodeoxycholic acid but not cholic acid is elevated.

The hypercholesterolemic effect of cholic acid was originally explained on the basis that it increased the absorption of exogenous and endogenous cholesterol. However, Friedman and Byers ('51) and Pihl ('55) have advanced evidence to show that exogenous bile acids often exert no effect on cholesterol absorption while causing a marked elevation of plasma cholesterol.

More recently it has been demonstrated in intact animals, that cholic acid reduces both rate of synthesis and mobilization of liver cholesterol (Beher and Baker, '58; Beher and Anthony, '58; Beher et al., '59). Further, Bergström and Danielsson ('58) have shown that it is the feedback of bile acid conjugates in the portal blood that controls the further oxidation of cholesterol to bile acids in the liver. A similar inhibition has also been shown to occur in an *in vitro* system (Whitehouse and Staple, '59).

There are at least two pathways of oxidation of cholesterol to bile acids in the animal organism (Bergström, '55; Siperstein and Chaikoff, '55). It has been demonstrated (Bergström, '55; Bergström and Lindstedt, '56; Lindstedt, '57) that no compound containing a carboxyl group in the side chain, with the exception of deoxycholic acid (Bergström et al., '53a),[2] is converted in the mammalian body to cholic acid; i.e., in the production of cholic acid the hydroxylation of the nucleus must occur before the oxidation of the side chain. On the other hand, lithocholic acid (Bergström et al., '53b) and chenodeoxycholic acid (Bergström and Sjoväll, '54; Matschiner et al., '57; Mahowald et al., '57) are each oxidized to more polar acids, none of which are identical with cholic acid. Some of these products have been identified as isomers of hyocholic acid, (3α,6α,7α) trihydroxycholanic acid (Hsia et al., '58a, b).[3]

In view of the many systems of checks and counterchecks found in the animal organism, it seemed logical to look for factors which increased or decreased the production of cholic acid or for factors which have an effect directly opposite to that of cholic acid. Recorded here are observations made in the course of an investigation of the effect of various bile acids on the plasma cholesterol concentration of the mouse.

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[1] Since the preparation of this manuscript hyodeoxycholic acid has been reported to reverse the effect of cholic acid in female mice and to prevent cholesterol accumulation in the liver and carcass (Beher, W. J., G. D. Baker and W. T. Anthony 1960 Effect of cholic and hyodeoxycholic acids on metabolism of exogenous cholesterol in mice. Proc. Soc. Exp. Biol. Med., *103:* 385).

[2] Matschiner, J. T., R. Richter, W. H. Elliot and E. A. Doisy, Jr. 1954 Metabolic studies of carboxyl-labeled C^{14}-desoxycholic acid. Federation Proc., *13:* 261 (abstract).

[3] Perry, G., and E. A. Doisy, Jr. 1959 Metabolism of lithocholic acid. Federation Proc., *18:* 301 (abstract).

EXPERIMENTAL

The experimental procedure is similar to that reported earlier (Howe and Bosshardt, '60). Male weanling mice were housed individually, 8 per group, and allowed to feed ad libitum. The basal diet consisted of casein, glucose, salt mixture, cellulose, and a vitamin addendum was supplemented at the expense of glucose. In many of the experiments a hydrogenated coconut oil,[4] was used as a dietary supplement after it was found that this substance enhanced the hypercholesterolemic effect of the bile acids.

At the end of a 12-day period the mice were weighed and decapitated after light chloroform anesthesia in order to obtain a pooled blood sample from each group of 8 animals. Plasma total cholesterol was determined by the method of Abell et al., ('52).

RESULTS

Some of the data obtained using the bile acids[5] and bile acid derivatives in amounts equivalent to 0.1% of cholic acid with 1% of cholesterol and 10% of hydrogenated coconut oil are recorded in table 1. The structures of the bile acids involved in this study are shown in figure 1.

Taurocholic acid appears to be somewhat less hypercholesterolemic than unconjugated cholic acid. This agrees with the previously reported finding that taurine lowers plasma cholesterol in the Cebus monkey (Portman and Mann, '55) and in the hypercholesterolemic rat (Hermann, '59). We were able to confirm this observation in the mouse and shall report our results in a later communication. Of considerable interest also is the observation that acetylation of methyl cholate in the 3 and 7 positions renders it completely inactive. The most striking observation obtained from this study, however, was that lithocholic acid was not only inactive in elevating plasma cholesterol but actually appeared to reduce it below the control level. In view of variations previously reported (Howe and Bosshardt, '60) and indicated here, these differences can be considered significant.

In view of the observations shown in table 1, it was logical to test lithocholic

[4] Hydrol, Durkee Famous Foods, Chicago.

[5] The cholic acid used in these studies was obtained from Nutritional Biochemicals Corporation. Its infrared absorption spectrum deviated slightly from that of a highly purified sample in the Merck standard files. However, by reversed phase chromatography no difference could be noted. The glycocholic acid, methylcholate, ethylcholate, methyldeoxycholate, methyl-3, 7-diacetylcholate, and chenodeoxycholic acid were prepared in the Merck Research Laboratories; the deoxycholic acid, lithocholic acid and hyodeoxycholic acid were purchased from Nutritional Biochemicals Corporation and the taurocholic acid from Wilson Laboratories. The infrared spectrum of none of these samples deviated from expectation. Finally the infrared absorption spectrum of the dehydrocholic acid obtained from Mann Research Laboratories showed some OH absorption and must be considered somewhat impure.

TABLE 1

Effect of bile acids on plasma cholesterol in the mouse when incorporated into the diet at levels equivalent on a molecular basis to 0.1% of cholic acid

Compound	Number of tests 8 animals/test	Av. plasma cholesterol[1]	Av. weight gain, 12 days
		mg/100 ml	*gm*
None	10	146 ± 16.8[2]	10.8
Cholic acid	10	258 ± 28.6	8.8
Taurocholic acid	5	185 ± 11.1	9.5
Glycocholic acid	5	231 ± 17.9	9.5
Deoxycholic acid	3	237 ± 9.7	10.7
Lithocholic acid	2	106 ± 2.5	10.6
Dehydrocholic acid	2	263 ± 1.0	9.9
Methyl cholate	4	243 ± 17.5	8.8
Ethyl cholate	2	243 ± 5.0	10.0
Methyl deoxycholate	3	231 ± 19.4	9.5
Methyl 3,7-diacetylcholate	2	151 ± 3.0	8.8

[1] In this and subsequent tables each cholesterol determination was made on a pooled blood sample from the 8 animals in each group.

[2] Standard error.

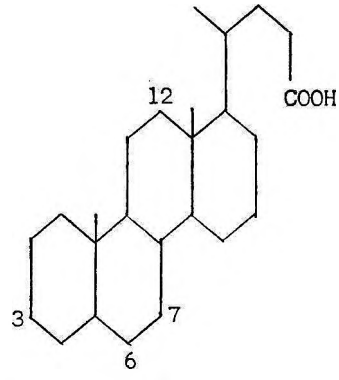

Fig. 1 Cholanic acid.
Lithocholic acid
—3α-hydroxycholanic acid
Deoxycholic acid
—3α, 12α-dihydroxycholic acid
Chenodeoxycholic acid
—3α, 7α-dihydroxycholanic acid
Hyodeoxycholic acid
—3α, 6α-dihydroxycholanic acid
Cholic acid
—3α, 7α, 12α-trihydroxycholanic acid
Hyocholic acid
—3α, 6α, 7α-thihydroxycholanic acid
β-Muricholic acid
—3α, 6β, 7β-trihydroxycholanic acid
Dehydrocholic acid
—3,7,12-triketocholanic acid

acid under a variety of conditions. It seemed likely that the most striking results might be obtained in animals receiving cholic acid and this system was used first with the results shown in table 2.

From these data lithocholic acid obviously counteracts the hypercholesterolemic effect of cholic acid. Accordingly a series of experiments was carried out to study the effect of lithocholic acid on other diets. The results are shown in table 3.

Analysis of variance of these data indicates that lithocholic acid especially at 0.2 to 0.4% of the diet ($P \leqq 0.05$) depresses the plasma cholesterol concentration of mice maintained with adequate purified diets.

It seemed desirable to determine whether lithocholic acid is effective against other bile acids which were shown to elevate plasma cholesterol. The results of experiments designed to check this point are shown in table 4. The basal diet again was supplemented with 10% of hydogenated coconut oil and 1% of cholesterol. Lithocholic acid can be seen to be effective in every case.

By means of radiolabeling techniques, lithocholic acid has been shown to be metabolized in the bile fistula rat to three more polar compounds (Bergström et al., '53b). In the surgically-jaundiced rat one of the products of metabolism has been identified as β-muricholic acid, 3α,6β,7β-trihydroxycholanic acid.[6] In a like manner, chenodeoxycholic acid and hyodeoxycholic acid (Hsia et al., '58a) have been shown to have muricholic acid as a common metabolic product. It is within the realm of possibility that lithocholic acid may exert its anti-cholic acid effect indirectly through muricholic acid. This hypothesis is supported to some extent by the finding of the St. Louis group that β-muricholic acid was found in the bile of rats fed laboratory chow, but not in the bile of those receiving a purified diet (Matschiner et al., '57). In our observations rats receiving chow have

[6] See footnote 3.

TABLE 2

Effect of lithocholic acid on concentration of plasma cholesterol of mice receiving 0.1% of cholic acid and 1.0% of cholesterol in the diet (12-day experiment)

Dietary supplement	Percentage of dietary lithocholic acid					
	0.0		0.1		0.5	
	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain
	mg/100 ml	*gm*	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*
10% Hydrogenated coconut oil[1] (3)[2]	236	7.6	216	7.2	131	5.9
15% Mixed fat (1)	182	7.6			138	7.6
5% Linseed oil (1)	206	12.6	146	12.8	136	10.3

[1] Hydrol.
[2] Figures in parentheses indicate number of groups of 8 animals each used at each level of lithocholic acid.

TABLE 3

Plasma cholesterol and weight gain as influenced by lithocholic acid and various fats

Fat supplement	Percentage of lithocholic acid									
	0.0		0.05		0.1		0.2		0.4	
	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain	Plasma cholesterol	Weight gain
	mg/100 ml	*gm*	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*	*mg/100 ml*	*gm*
15% Mixed fat	139	13.9	117	12.9	113	12.8	113	13.0	100	9.1
5% Hydrogenated coconut oil[1]	107	10.8	129	10.7	132	9.1	103	11.2	99	9.1
5% Corn oil	114	12.4	120	12.6	114	11.5	95	12.5	80	6.3
None	100	10.5	107	10.6	89	10.0	77	9.7	95	6.7
5% Linseed oil	84	11.2	99	11.7	91	12.0	87	10.7	92	9.9
5% Cod liver oil	84	12.5	87	13.6	82	12.7	66	11.6	64	8.7

[1] Hydrol.

shown consistently lower concentrations of plasma cholesterol than those ingesting a purified diet. It might therefore be inferred that the presence of muricholic acid in the chow-fed rats may be responsible for their lower plasma cholesterol concentrations. At any rate, it seemed worthwhile to check the other precursors of β-muricholic acid, chenodeoxycholic and hyodeoxycholic acids for anti-cholic acid effect.

The results of the tests with chenodeoxycholic acid are shown in tables 5 and 6. From these data it is apparent that chenodeoxycholic acid has much the same effect as lithocholic acid in counteracting the hypercholesterolemic effect of cholic acid.

A comparison of the effect of hyodeoxycholic acid with lithocholic and chenodeoxycholic acids is shown in table 7. These observations show that hyodeoxycholic acid is the most effective of the three bile acids in counteracting the hypercholesterolemic effect of cholic acid.

Some of the dietary supplements, especially at the higher levels used, caused a definite growth inhibition. The results of experiments which show that this inhibition *per se* was not responsible for the depressed plasma cholesterol concentrations are recorded in an earlier publication (Howe and Bosshardt, '60). In addition, a group of weanling mice receiving the basal diet with cholesterol and cholic acid were restricted to 2 gm of food daily which limited their growth to approximately 2 gm in 12 days. The plasma cholesterol concentration of pooled sample from these animals was 307 mg/100 ml. Values obtained by assay of pooled samples from animals similarly restricted with comparable rates of growth receiving in addition 0.4% of lithocholic acid and 0.4% of hyodeoxycholic acid were 113 and 135 mg/100 ml, respectively.

DISCUSSION

In light of the observation that lithocholic and chenodeoxycholic acids, both of which appear to arise in the liver, counter the hypercholesterolemic effect of cholic acid it is tempting to assume that a balance of the various bile acids may be a body mechanism for the control of plasma cholesterol. A variety of factors might in-

TABLE 4

Effect of lithocholic acid on concentration of plasma cholesterol (mg/100 cm³) of mice receiving 0.1% of various bile acids added to a basal diet containing 10% of hydrogenated coconut oil and 1% of cholesterol (12-day experiment)

Dietary bile acid	Percentage of lithocholic acid		
	0.0	0.2	0.4
Cholic acid	315(7.7)[1]	144(8.0)	143(7.4)
Taurocholic acid	213(8.6)	139(9.4)	118(7.5)
Glycocholic acid	296(7.6)	159(7.8)	122(9.2)
Deoxycholic acid	255(7.1)	157(6.6)	120(7.0)
Methyl cholate	259(6.8)	170(7.5)	135(5.7)
Dehydrocholic acid	262(8.4)	138(7.0)	118(5.0)

[1] Figures in parentheses indicate weight gain in grams.

TABLE 5

Comparison of effect of lithocholic and chenodeoxycholic acids on plasma cholesterol concentration of mice receiving 0.1% of cholic acid and 1.0% of cholesterol in 12-day test

Dietary supplement	Percentage of bile acid added			
	Lithocholic acid			Chenodeoxycholic acid
	0.0	0.1	0.5	0.1
None	234(9.6)[1]	169(10.1)	106(8.7)	153(9.6)
5% Mixed fat	195(12.4)	187(12.7)	142(6.6)	169(12.4)
5% Cod liver oil	158(11.0)	123(11.6)	116(7.2)	144(12.4)
5% Hydrogenated coconut oil[2,3]	250(10.1)	160(9.8)	123(5.4)	169(8.4)

[1] Figures in parentheses show average weight gain in grams.
[2] Hydrol.
[3] All figures are an average of values obtained with two groups of 8 mice each.

TABLE 6

Comparison of anti-cholic acid effect of lithocholic acid and chenodeoxycholic acid in 4-day test

Dietary supplement	Plasma cholesterol
	mg/100 ml
5% Hydrogenated coconut oil[1]	136
5% Hydrogenated coconut oil, 0.1% cholic acid, 1% cholesterol	177
5% Hydrogenated coconut oil, 0.1% cholic acid, 1% cholesterol + 0.1% chenodeoxycholic acid	147
5% Hydrogenated coconut oil, 0.1% cholic acid, 1% cholesterol + 0.1% lithocholic acid	134
5% Hydrogenated coconut oil, 0.1% cholic acid, 1% cholesterol + 0.5% lithocholic acid	128

[1] Hydrol.

fluence this balance and in so doing alter the plasma cholesterol concentration. For example, Gordon et al., ('57a, b) have reported an increase in total bile acid excretion in man after the administration of sunflower seed oil with its high content of linoleic acid and this result has been confirmed by Haust and Beveridge, ('58) with linoleic acid alone, but with the further observation that this increase could be accounted for by an increase in the dihydroxycholanic acid fraction. Eriksson, ('57) has found that a thyroid-active material, which is known to influence plasma choles-

TABLE 7

Concentrations of plasma cholesterol (mg/100 ml) of mice receiving 0.1% of cholic acid and 1.0% of cholesterol in combination with graded levels of selected bile acids in 12-day test

Dietary supplement	Percentage of bile acid in diet					
	0.0	0.025	0.05	0.1	0.2	0.4
Exp. 1, 10% Hydrogenated coconut oil[1]						
Lithocholic acid	288(6.2)[2]	250(6.0)	224(8.7)	186(6.7)	177(7.1)	175(6.0)
Hyodeoxycholic acid	—	224(5.6)	192(8.2)	177(8.2)	145(7.1)	110(5.9)
Chenodeoxycholic acid	—	257(7.0)	214(7.2)	219(8.8)	191(5.9)	—
Exp. 2, 0.5% Linoleic acid						
Chenodeoxycholic acid	246(10.3)	—	—	—	171(10.5)	—
Hyodeoxycholic acid	—	—	—		128(10.3)	—

[1] Hydrol.

[2] Figures in parentheses indicate weight gain in grams.

terol in some species, increases the ratio of chenodeoxycholic acid to cholic acid in the rat.

On the other hand, hyodeoxycholic as far as is known arises only through bacterial action on hyocholic acid in the intestinal tract of the pig (Bergström et al., '59). It is possible that lithocholic and chenodeoxycholic acids may exert their effects through bacterial transformation to unidentified products. Germ-free rats have been found to possess higher concentrations of blood cholesterol than normal controls despite the fact that Norman and Sjoväll ('58) could find no evidence that the total quantity of unabsorbable transformation products was increased in the latter.

The observation that taurocholic is less hypercholesterolemic than unconjugated cholic acid and that taurine decreases the hypercholesterolemic effect of cholic acid may open an attractive avenue of research. It is known that enzyme systems exist for conjugation and hydrolysis of cholic acids. Factors which influence these reactions may be of importance in regulating blood cholesterol. In a like manner, that acetylation of cholic acid neutralizes its activity may also be important. While no similar enzyme systems are known for this acetylation, that bile salts are required for the esterification of cholesterol with fatty acids has been established (Swell and Treadwell, '50). It has been suggested that this reaction is brought about by a primary esterification of the bile salt followed by transesterification. More than molar quantities of bile salt are known to inhibit cholesterol esterification (Swell et al., '53). Again if such a system existed, a study of the factors which influence it could be important.

SUMMARY

From studies carried out in the weanling mouse, it was concluded that:

1. Taurocholic acid is less hypercholesterolemic than cholic acid.

2. Acetylation of methyl cholate in the three and 7 poistions eliminates its hypercholesterolemic action.

3. Lithocholic acid exerts a hypocholesterolemic effect and neutralizes the action of hypercholesterolemic bile acids.

4. Chenodeoxycholic acid has much the same action as lithocholic acid while hyodeoxycholic is more effective than lithocholic or chenodeoxycholic acid in neutralizing the hypercholesterolemic effect of cholic acid.

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LITERATURE CITED

Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

Beher, W. T., and W. L. Anthony 1958 Liver cholesterol metabolism in mice as effected by dietary β-sitosterol and cholic acid. Proc. Soc. Exp. Biol. Med., *99:* 356.

Beher, W. T., and G. D. Baker 1958 Effect of dietary bile acids on *in vivo* cholesterol metabolism in the rat. Ibid., *98:* 892.

Beher, W. T., G. D. Baker and W. L. Anthony 1959 Effect of dietary cholic acid on *in vivo* cholesterol metabolism. Ibid., *100:* 3.

Bergström, S. 1955 Formation and metabolism of bile acids. Record Chem. Progr., *16:* 63.

Bergström, S., and H. Danielsson 1958 On the regulation of bile acid formation in the rat liver. Acta Physiol. Scand., *43:* 1.

Bergström, S., and S. Lindstedt 1956 The formation of cholic acid from 3α, 7α-dehydroxycoprostane in the rat. Biochem. Biophys. Acta, *19:* 556.

Bergström, S., and J. Sjoväll 1954 Occurrence and metabolism of chenodesoxycholic acid in the rat. Acta Chem. Scand., *8:* 611.

Bergström, S., H. Danielsson and A. Goransson 1959 On the bile acid metabolism in the pig. Bile acids and steroids 81. Ibid., *13:* 776.

Bergström, S., M. Rottenberg and J. Sjoväll 1953 Über der stoffvechsel der cholsaüre und desoxycholsaüre in der ratte. X. Metteilung über steroide und gallensaüren. Ztschr. Physiol. Chem., *295:* 278.

Bergström, S., J. Sjoväll and J. Voltz 1953b Metabolism of lithocholic acid in the rat. Acta Physiol. Scand., *30:* 22.

Byers, S. O., M. Friedman and F. Michaelis 1950 Observations concerning the production and excretion of cholesterol in mammals. J. Biol. Chem., *184:* 71.

Eriksson, S. 1957 Influence of thyroid activity on excretion of bile acids and cholesterol in the rat. Proc. Soc. Exp. Biol. Med., *94:* 582.

Friedman, M., and S. O. Byers 1951 Cholic acid: An adequate stimulus for hypercholesteremia in the normal fasting rat. Proc. Soc. Exp. Biol. Med., *78:* 528.

Friedman, M., S. O. Byers and R. H. Rosenman 1952 The accumulation of serum cholate and its relationship to hypercholesteremia. Science, *115:* 313.

Gordon, H., B. Lewis, L. Eales and J. F. Brock 1957a Effect of different dietary fats on the fecal end-products of cholesterol metabolism. Nature, *180:* 923.

——— 1957b Dietary fat and cholesterol metabolism. Lancet, *273:* 1299.

Haust, H. L., and J. M. R. Beveridge 1958 Supplement to International Abstracts of Biological Sciences, International Congress of Biochemistry, New York, p. 204.

Hermann, R. G. 1959 Effect of taurine, glycine and β-sitosterols on serum and tissue cholesterol in the rat and rabbit. Circ. Res., *7:* 224.

Howe, E. E., and D. K. Bosshardt 1960 Antibiotics and plasma cholesterol in the mouse. J. Nutrition, *72:* 368.

Hsia, S. L., J. T. Matschiner, T. A. Mahowald, W. H. Elliot, E. A. Doisy, Jr., S. A. Thayer and E. A. Doisy 1958a Bile acids. VII. Structure of acid I. J. Biol. Chem., *230:* 573.

——— 1958b Bile acids. X. Characterization and partial synthesis of acid IV. Ibid., *230:* 597.

Lindstedt, S. 1957 The formation of bile acids from 7 α-hydroxycholesterol in the rat. Acta Chem. Scand., *11:* 417.

Mahowald, T. A., J. T. Matschiner, S. L. Hsia, R. Richter, E. A. Doisy, Jr., W. H. Elliot and E. A. Doisy 1957 Bile acids. II. Metabolism of deoxycholic acid-24-C^{14} and chenodeoxycholic acid-24-C^{14} in the rat. J. Biol. Chem., *225:* 781.

Matschiner, J. T., T. A. Mahowald, W. H. Elliot, E. A. Doisy, Jr., S. L. Hsia and E. A. Doisy 1957 Bile acids. I. Two new acids from rat bile. Ibid., *225:* 771.

Norman, A., and J. Sjoväll 1958 On the transformation and enterohepatic circulation of cholic acid in the rat. Ibid., *233:* 872.

Pihl, A. 1955 The effect of bile acids on cholesterol absorption, deposition and synthesis in the rat. Acta Physiol. Scand., *34:* 206.

Portman, O. W., and G. V. Mann 1955 The disposition of taurine-S^{35} and taurocholate-S^{35} in the rat: dietary influences. J. Biol. Chem., *213:* 733.

Siperstein, M. D., and I. L. Chaikoff 1955 Conversion of cholesterol to bile acids. Federation Proc., *14:* 767.

Swell, L., and C. R. Treadwell 1950 Cholesterol esterases. J. Biol. Chem., *182:* 479.

Swell, L., H. Field, Jr. and C. R. Treadwell 1953 Role of bile salts in activity of cholesterol esterase. Proc. Soc. Exp. Biol. Med., *84:* 417.

Whitehouse, M. W., and E. Staple 1959 Regulation of cholesterol oxidation by liver *in vitro*. J. Biol. Chem., *101:* 439.

Wysocki, A. P., O. W. Portman and G. V. Mann 1955 Bile acids in blood: methods and their application to studies of experimental atherosclerosis in monkeys. Arch. Biochem. Biophys., *59:* 213.

INVITATIONS FOR NOMINATIONS FOR 1961 AMERICAN INSTITUTE OF NUTRITION AWARDS AND FELLOWS

Nominations are now being invited for the 1961 A. I. N. awards and fellowships.

Nominations for the 1961 *Borden Award in Nutrition* must be submitted by December 1, 1960, to Dr. G. V. Mann, Vanderbilt University Hospital, Nashville, Tennessee.

Nominations for the 1961 *Osborne and Mendel Award* are due also by December 1, 1960, and should be sent to Dr. J. S. Dinning, Department of Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas.

The deadline for receipt of nominations for *A. I. N. Fellows* is January 1, 1961. These should be sent to Dr. W. D. Salmon, Department of Animal Husbandry and Nutrition, Alabama Polytechnic Institute, Auburn, Alabama.

Full details of the rules for these awards and lists of former recipients are given in the August 1960 issue of *The Journal of Nutrition.*

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