LIVER NECROSIS AND ALTERED FAT COMPOSITION IN VITAMIN E-DEFICIENT SWINE [1]

E. L. HOVE AND H. R. SEIBOLD

Department of Animal Husbandry and Nutrition, Agricultural Experiment Station and the Department of Pathology, School of Veterinary Medicine, Alabama Polytechnic Institute, Auburn

FOUR FIGURES

(Received for publication December 6, 1954)

A statement that swine require dietary vitamin E can not be made without contradiction and argument. Data denying a demonstrable requirement were offered by Hanson and Hathaway ('48, '49) and by Bratzler, Loosli, Krukovsky and Maynard ('50). Swine fed from weaning on synthetic diets with 25% casein showed no abnormality in growth or reproduction, nor any pathological alterations attributable to the lack of vitamin E. In neither case did the diet contain cod-liver oil or other highly unsaturated fats.

On the other hand a yellowish-brown discoloration of adipose tissue of swine has necessitated condemnation of carcasses at commercial slaughter, and this condition has been produced experimentally. One of the causes has been established to be a combination of too little dietary vitamin E in the presence of an excessive amount of unsaturated fatty materials, such as marine fish scraps, cod-liver oil, linseed oil meal, or horse meat with its exceptionally high content of lino-

[1] Published with the approval of the Director of the Agricultural Experiment Station, and the Committee on Publications of the School of Veterinary Medicine as paper number 503. This work was aided by a grant from the National Vitamin Foundation. Appreciation is expressed to Merck and Company and to A. E. Stalay and Company for supplies of vitamins.

lenic acid (Davis and Gorham, '54; Robinson and Coey, '51; Beadle, Wilder and Kraybill, '48). Different from the "yellow fat" disease of mink (Lalor, Leoshke and Elvehjem, '51) with its extensive pathology and fatal outcome, the yellow fat condition of hogs was not accompanied by pathological changes elsewhere in the carcass.

Evidence of a positive need for vitamin E was offered by Adamstone, Krider and James ('49). Poor reproductive performance was noted in 6 sows on a diet of wheat flour, yeast, and minerals, with casein added at a 4% level. Two of these died during or shortly after pregnancy. In all cases abnormalities in the liver were noted, consisting of irregular distribution of fat, frequent increase in the thickness of the connective tissue bands between liver lobules, and in one case a severe hemorrhagic condition of the liver. The surviving offspring had muscular weakness and incoordination. Six control sows, receiving tocopherol, were normal, as were their offspring.

Carpenter and Lundberg ('49) reported that "baby pig" disease could be largely prevented by dosing the sows with vitamin E (1 gm daily for 30 days). The number of young surviving to weaning in the untreated lot was 22%, while in the treated lot it was 63%. "Baby pig" disease is a non-infectious fatality occurring a few days after birth and characterized by hypoglycemia.

The name of *Hepatosis dietetica* was given by Obel ('53) to a fatal condition occurring with high frequency in the swine herds of Sweden and other North European countries. This non-infectious disease occurs suddenly, and with no pre-mortal weight loss or malaise. Animals that appear normal become sluggish, pass into a coma, and die within a few hours. This disease may appear a few weeks after birth, but reaches its maximum incidence at about 6 to 8 weeks. The incidence gradually declines up to the 16th week of life. The pathology is characterized by extensive necrosis of the liver in all cases. Less frequently there occur ulceration of the stomach and colon, waxy degeneration of voluntary muscle, pulmonary

edema, hemorrhage in the lungs and hemorrhage in the lymph nodes.

A fatal disease apparently identical with the naturally occurring condition was produced experimentally by Obel ('53) and shown to be preventable by oral doses of α-tocopheryl acetate. The diet fed to weanling pigs consisted of starch, minerals, yeast (as the sole source of the 9% protein in the diet), and 6% cod-liver oil.

In rats, the disease of liver-necrosis was shown by Schwarz ('44) to be due to a vitamin E deficiency combined with inadequate protein, such as yeast. Matet, Matet and Fridenson ('49) used soybean meal as the inadequate necrogenic protein, while Hove, Copeland and Salmon ('49) used low (10%) casein levels to produce necrosis and lung hemorrhage. Dam and Granados ('51) showed that cod-liver oil was a necessary stress factor in the low casein diet to produce the typical pathology. The level of tocopherol required to prevent pathology varied directly with the severity of the stress conditions of the diet; however, this level is in all cases at least 5 mg per kilogram of body weight. This corresponds to 0.2 gm of tocopherol daily for a 100-lb. hog.

The report of Obel became available during the course of the experiments reported in the present paper, the data of which are in complete agreement with her results.

EXPERIMENTAL

The basal diet (H83) was composed as follows: soybean meal defatted, 12%; sucrose, 75%; mineral mixture,[2] 5%; lard, 6% and cod-liver oil, 2%. Pure vitamins were added to give these levels per gram of diet; thiamine, riboflavin, pyridoxine, 3 μg each; calcium pantothenate, 15 μg; niacin, 30 μg; 2-methyl napthoquinone, 0.5 μg; inositol, 0.1 mg; and choline, 1 mg. Vitamin B_{12} and folacin were omitted since some evidence (Hove and Hardin, '51) has indicated that the absence of these vitamins accentuates the vitamin E deficiency in rats.

[2] Salmon, W. D. J. Nutrition, *33:* 155, 1947.

Two litters of Hampshire hogs, with 6 animals per litter, have been fed the experimental diets. The first litter (experiment I) was born August 11, 1953. The sow was on pasture-dry lot combination and farrowed on concrete. The young were weaned at 6 weeks and penned in two groups of three animals each in concrete, half-covered outdoor pens. The animals were hand-fed twice daily, watered daily, and the pens washed down with a hose twice weekly. The diets were mixed twice weekly. One group of three pigs received the vitamin E-low diet, H83; the other group of three pigs was fed the control diet, of the same composition, but with the addition of 0.01% dl-α-tocopheryl acetate. With an average feed intake of 1.5 kg daily, this corresponds to 150 mg of the tocopherol. This low level was subsequently doubled. The mixed ration was not refrigerated.

The second litter (experiment II) was born on April 7, 1954. The sow was in a concrete pen on a dry-lot ration of corn, soybean meal, alfalfa leaf meal and minerals. The young were weaned at 4 weeks, and the 6 pigs set up in two lots as in experiment I. The average weaning weight in experiment I was 35 lbs., while the earlier-weaned pigs of experiment II averaged 23 lbs.

The surviving animals of experiment I were slaughtered after 187 days on the experimental diets. Appropriate tissues were preserved for histological examination, and samples of the liver and ham muscle taken for unsaturated fatty acid determinations by the method of Herb and Riemenschneider ('53). The surviving animals of experiment II were slaughtered after 86 days for pathological examination. Plasma and tissue tocopherols were determined by the method previously indicated (Hove, '53).

RESULTS

The rate of growth of the pigs on the synthetic diet was about 0.65 lb. per day (table 1). This is remarkable growth, considering that the protein level in the diet was only 6%, and all of this furnished by soybean meal. Normal growth in this type of hog is considered to be about 1.2 lb. per day.

TABLE 1

Growth, plasma tocopherol, and pathological effects of vitamin E deficiency in swine on low-protein diets

EXP. NO.	DIET	PIG		DAYS ON DIET	BODY WEIGHT			PLASMA TOCOPHEROL	PATHOLOGICAL EFFECTS	
		No.	Sex		Start	End	Gain		Death	Liver damage
					lb.	*lb.*	*lb./day*	*μg %*		
I	H83	30	M	187	33	154	0.65	132	Killed	Post-necrotic cirrhosis
		31	M	127	35	96	0.48	101	Died	Hemorrhagic necrosis
		32	F	187	34	184	0.80	115	Killed	Slight cirrhosis
						Av.	0.64			
	H83*E*	34	F	187	30	170	0.75	180	Killed	Negative
		35	M	187	38	140	0.55	240	Killed	Negative
		36	M	96	34	109	0.78	160	Died	Hemorrhagic necrosis
						Av.	0.69			
II	H83	163	F	86	29	96	0.78	0	Killed	Post-necrotic cirrhosis
		164	F	39	19	40	0.57	?	Died	Hemorrhagic necrosis
		165	M	34	24	42	0.60	?	Died	Hemorrhagic necrosis
						Av.	0.65			
	H83*E*	160	M	86	22	101	0.92	0	Killed	Negative
		161	F	86	24	69	0.53	108	Killed	Negative
		162	F	86	19	62	0.50	120	Killed	Negative
						Av.	0.65			

The tocopherol supplement had no effect upon the growth rate (table 1).

Estimations of plasma tocopherol on the pigs near the end of the experiments (table 1) revealed that the animals on the control diet H83E were not receiving sufficient tocopherol in their diet to maintain adequate blood levels of this vitamin. Two factors may be involved in the low plasma levels. First, the hog appears to require more dietary vitamin E than certain other species to maintain the adequate level of this vitamin. This is evident from the work of Bratzler et al. ('50), who fed different levels of tocopherol to swine and found so-called normal levels only when about 1 gm per day per 100 pound of body weight was fed. Dilution of the tocopherol in the excessive quantities of body fat in this species may play a part in this phenomenon. Second, in the present experiments the tocopherol supplement was mixed with the diet containing the cod-liver oil and stored at atmospheric temperatures from one to three days before feeding. The atmospheric temperature in experiment I was relatively low (October to March). However, the atmospheric temperature in experiment II was very high (May, June and July). Undoubtedly a considerable amount of the tocopherol added to the diet was destroyed by these conditions, and this fact is reflected in the near absence of plasma tocopherol in the animals of experiment II (table 1).

No outward symptoms of ill health were observed and the general condition of the animals appeared excellent. No diarrhea was evident at any time. Pig 30, of experiment I, was stiff-legged for some time prior to death, but analysis of his urine showed no creatinurea. A total of 4 pigs died. There was no pre-mortal weight loss or malaise. The appearance of the animals up to the day of death was excellent in all cases. At slaughter, gross liver damage was evident in all pigs on diet H83 (table 1).

The composition of liver and muscle fat of the 4 surviving animals of experiment I is given in table 2. Each tissue was assayed in duplicate, so the values given in the table are

averages of 4 determinations. In the absence of dietary vitamine E the fat content of liver decreased, while the fat content of muscle increased. The fat composition values of table 2 show that, in comparison with the added-vitamin E controls, the vitamin E-deficient hogs had less linoleic, arachidonic and pentaenoic acids in muscle fat; the same changes were seen in liver fat, except for the arachidonic acid level; conjugated dienes were higher in these fats. No tocopherol was detected in any samples. The changes in fat content and fat composi-

TABLE 2

Content and composition of the fat from liver and muscle of vitamin E-deficient and control pigs on low-protein diets [1]

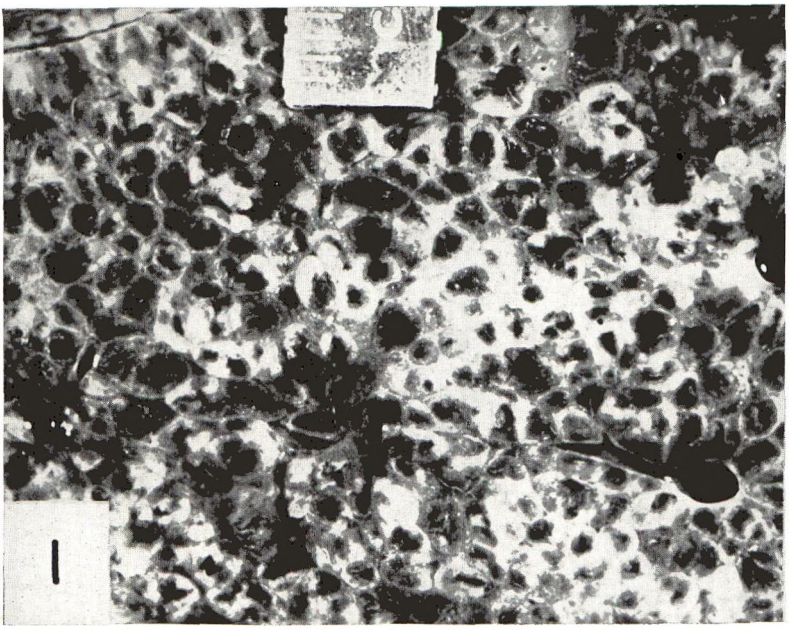
	LIVER		MUSCLE	
	No added E	Plus E	No added E	Plus E
Total fat of tissue (% wet basis)	1.56	2.31	6.33	3.76
Oleic acid (% of fat)	27.9	11.4	59.3	61.8
Linoleic acid (% of fat)	9.62	15.92	3.09	5.37
Linolenic acid (% of fat)	1.31	(— 0.34)	1.40	(— 0.33)
Arachidonic acid (% of fat)	6.54	5.78	1.36	2.19
Pentaenoic acid (% of fat)	9.96	13.98	0.72	1.14
Conj. dienes (% of fat)	1.49	0.71	0.31	0.27
Iodine number	97.5	99.7	79.1	79.6

[1] The column headings "No added E" and "Plus E" refer to the pigs fed diet H83 and H83E of experiment I.

tion have certain points of similarity with those of rats fed tri-o-cresyl phosphate with and without vitamin E (Hove, '53).

Pathology

The 4 hogs that died during the experiment showed severe acute hemorrhagic necrosis of the liver comparable to the *Hepatosis dietetica acuta* described by Obel ('53). Three of the hogs (31, 164, 165) were fed the vitamin E-deficient diet, and one (36) was fed the control diet, but died before the amount of DL, α-tocopheryl acetate was doubled. On gross examination the affected livers were extremely mottled due to



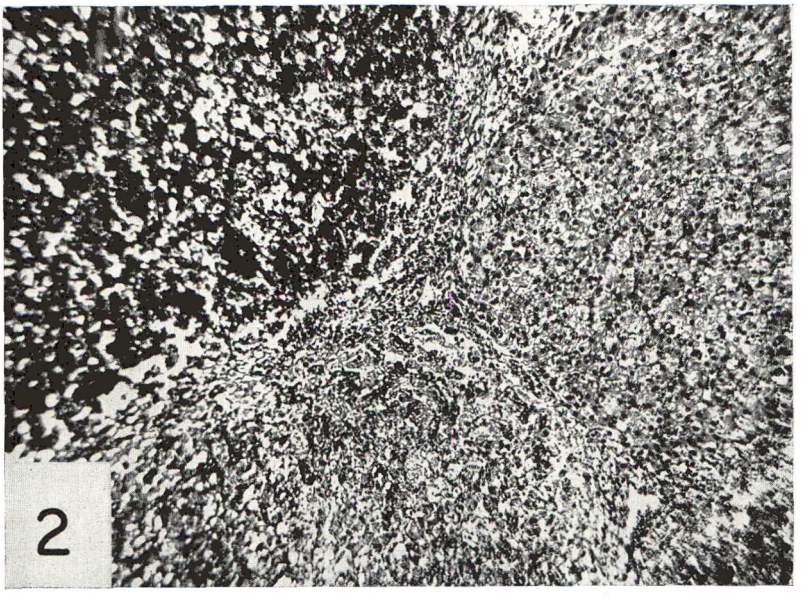


Fig. 1 Cross section of hog liver showing acute hemorrhagic necrosis.

Fig. 2 Histologic section of hog liver with acute hemorrhagic necrosis showing a portion of an affected (left) and non-affected (right) lobule. × 90.

the fact that some lobules were completely hemorrhagic, others were partially hemorrhagic, and a few appeared normal (fig. 1). Histopathological examination revealed total necrosis of the hepatic parenchyma with hemorrhage in many of the lobules; in some of the lobules the parenchyma had been partially or completely spared (fig. 2). The necrosis did not appear to be zonal in character. In lobules where the parenchyma had been partially spared, the necrotic area either was adjacent to the lobular border or extended from the central area in one or more directions towards the periphery. There was considerable variation in the amount of hemorrhage in the damaged areas which, no doubt, helped to accentuate the mottled appearance of the affected livers presented in gross examination. In one case (36) some of the severely damaged lobules had collapsed, and the remaining necrotic liver cells were undergoing calcification. The 4 hogs that died also showed hemorrhages in various locations including the lymph nodes, endocardium, epiglottis, lungs and gastrointestinal mucosa. These hemorrhages apparently occurred as a result of the severe liver damage.

Two hogs (30 and 163) that were slaughtered after being fed the vitamin E-deficient diet showed a post-necrotic cirrhosis comparable to the *Hepatosis dietetica chronica* described by Obel ('53). The word "cirrhosis" is used as defined by Lichtman ('49), i.e. — all sclerosed conditions of the liver, whether progressive or not, in which destruction of liver cells is associated with real or apparent increase of connective tissue. On gross examination the affected livers appeared to be studded with nodules which in reality were abnormally large lobules set off by atrophic, partially fibrosed lobules (fig. 3). In addition some lobules contained small reddish foci. Histopathological examination revealed a definite increase of fibrous tissue throughout the affected organs (fig. 4). In the most severely affected liver (30) some lobules were almost completely replaced by fibrous tissue. Other lobules in both livers were abnormally large from regeneration of parenchymal cells and were partially subdivided into lesser

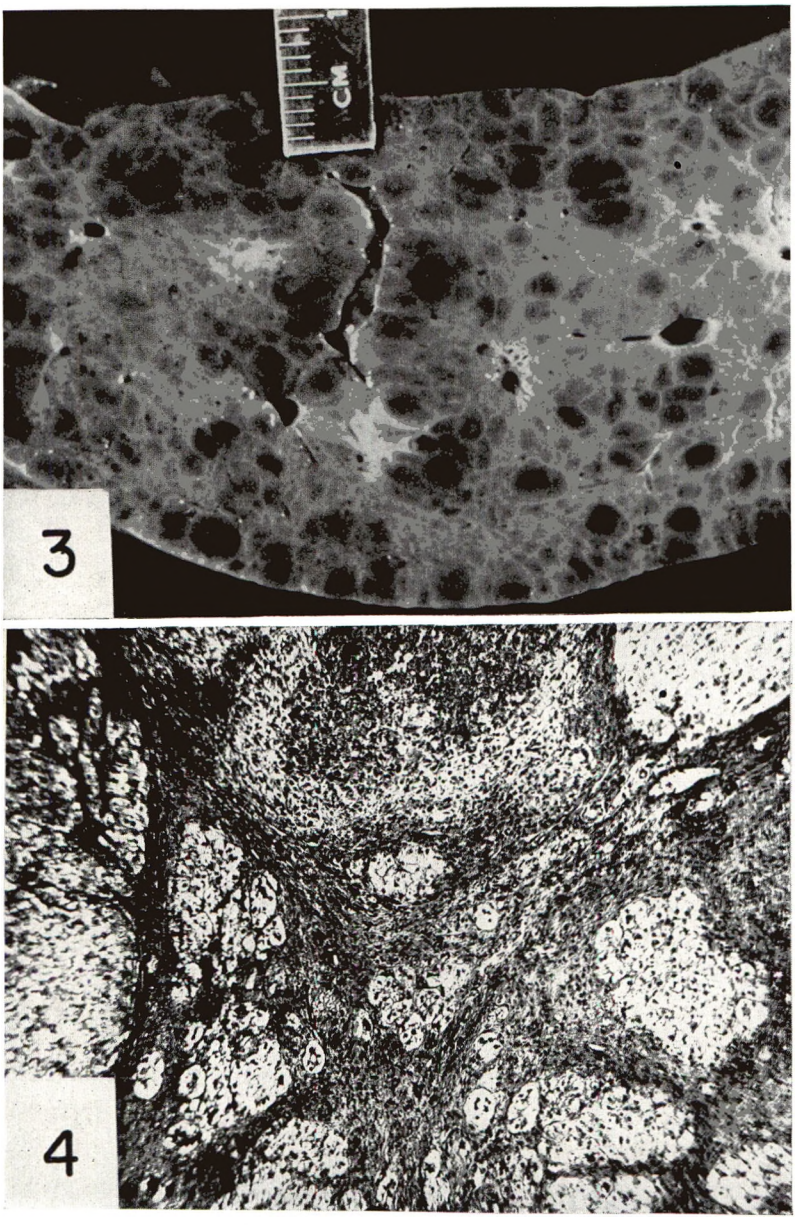


Fig. 3 Cross section of hog liver showing post-necrotic cirrhosis.

Fig. 4 Histologic section of hog liver with post-necrotic cirrhosis showing lobular atrophy and fibrosis. × 90.

units by bands of fibrous tissue projecting inward from the interlobular septa. Large foci of parenchymal necrosis, both with and without hemorrhage, were seen in some of the lobules. The presence of necrotic foci gave evidence of the post-necrotic character of the cirrhosis developing in these livers that had not been sufficiently damaged by acute hemorrhagic necrosis to cause death. In both livers some of the parenchymal cells had a clear or rarefied cytoplasm characteristic of cells in a state of protein depletion. There also was a slight amount of bile duct proliferation.

Only one hog (32) of the 6 fed the vitamin E-deficient diet failed to show well-marked pathological changes of the liver. This animal was slaughtered after 187 days of experimental feeding. There was a slight patchy increase of connective tissue along with some abnormal variation in the size of the hepatic lobules, but no necrosis.

One of the control animals (36) that died before the amount of vitamin E in the control diet was doubled showed acute hemorrhagic necrosis of the liver and is included in the 4 hogs first mentioned in the pathological description. The other 5 control hogs (34, 35, 160, 161, 162) failed to show either hemorrhagic necrosis of the liver or post-necrotic cirrhosis.

Ceroid pigment was found in the body fat of the two hogs (30, 32) that survived on the vitamin E-deficient diet in experiment I and were slaughtered. These animals had been fed the deficient diet for 187 days, which was more than twice as long as any of the hogs in experiment II.

Muscle tissue taken from 4 of the vitamin E-deficient hogs and 4 of the control hogs failed to show hyaline degeneration or necrosis.

DISCUSSION

Swine developed a fatal liver necrosis when fed a special diet, deficient in vitamin E, and containing cod-liver oil and a relatively low level of protein. Without cod-liver oil in this diet liver necrosis would not have occurred, in all probability, since Dam and Granados ('51) have shown the importance of this stress factor to development of the pathology. The

condition appeared to be identical with that described by Obel ('53), who also reported rather extensive occurrence under commercial and practical conditions on farms in the North European area. As far as now known, this disease does not occur in the United States. However, a somewhat similar condition known as "moldy corn" disease occurs in southern Alabama and Georgia. It is of interest that in this region it is recommended that stored corn be fumigated with carbon tetrachloride and other chlorinated hydrocarbons at concentrations of about 1:1000. Such fumigants are oxidative in nature (Hove, '53) and tend to destroy what little vitamin E exists in corn.

Corn, as well as soybeans and oats, is quite low in vitamin E. This little realized or appreciated fact contributes a good deal of confusion to the nutrition of vitamin E in farm animals. Hove and Hove ('44) showed that nearly all (90 to 95%) of the tocopherol of these feedstuffs occurred as the relatively biologically inactive γ-tocopherol. This finding has been repeatedly confirmed by other workers and has been reviewed by Brown ('52). A simple calculation shows that 1 kg of corn will supply only 6 mg of α-tocopherol, as vitamin E activity. (This value is obtained by ascribing 10% biological activity to the γ-tocopherol, Hove and Harris, '47.) Yet Bratzler et al. ('50) had to feed 10 times this amount of vitamin E to get any tocopherol into the blood plasma, and fed 250 times the amount furnished by a whole corn diet to get blood plasma values up to 750 μg%, a level which in other animals is considered to be about the normal value.

It is probable that a diet for swine based upon corn, and containing a stress factor such as cod-liver oil, and with no access to pasture or other source of vitamin E, would allow the development of the fatal liver necrosis described in this paper.

SUMMARY

A fatal liver necrosis developed in growing pigs fed a diet deficient in vitamin E, and containing 6% protein as furnished by soybean meal, and with 2% cod-liver oil. Of the 6 pigs fed

this diet from weaning, three died suddenly with massive acute hemorrhagic liver necrosis. Two of the survivors, when slaughtered, showed post-necrotic cirrhosis of the liver, but lower than normal liver fat. Muscle fat of vitamin E-deficient hogs had lesser concentrations of linoleic, arachidonic and pentaenoic acids than did that of added-vitamin E controls; liver fat showed similar changes except in arachidonic acid concentrations.

Five of the 6 animals in control lots on the same basal diet but supplemented with α-tocopheryl acetate survived to slaughter and failed to show appreciable liver damage.

ACKNOWLEDGMENT

Fat compositions and tocopherol analyses were performed by Zelda Hove, to whom appreciation is expressed.

LITERATURE CITED

ADAMSTONE, F. B., J. L. KRIDER AND M. F. JAMES 1949 Response of swine to vitamin E-deficient rations. Ann. N. Y. Acad. Sci., *52:* 260 .

BEADLE, B. W., O. H. M. WILDER AND H. R. KRAYBILL 1948 Study on the causes of "Yellow Fat" in hogs. Provisioner, Jan. 10; 26.

BRATZLER, J. W., J. K. LOOSLI, V. N. KRUKOVSKY AND L. A. MAYNARD 1950 Effect of the dietary level of tocopherols on their metabolism in swine. J. Nutrition, *42:* 59.

BROWN, F. 1952 The estimation of vitamin E. 2. Quantitative analysis of tocopherol mixtures by paper chromatography. Biochem. J., *52:* 523.

CARPENTER, L. E., AND W. O. LUNDBERG 1949 Effect of tocopherols on vitality of pigs in relation to "Baby Pig Disease." Ann. N. Y. Acad. Sci., *52:* 269.

DAM, H., AND H. GRANADOS 1951 The influence of certain substances on massive hepatic necrosis and lung hemorrhage in rats fed low-protein, vitamin E deficient diets. Acta pharmacol. et toxicol., *7:* 181.

DAVIS, C. L., AND J. R. GORHAM 1954 The pathology of experimental and natural cases of "Yellow Fat" disease in swine. Am. J. Veter. Resear., *15:* 55.

HANSON, L. E., AND I. L. HATHAWAY 1948 The fertility of boars fed a vitamin E deficient ration. J. Anim. Sci., *7:* 528.

———— 1949 Swine nutrition. Vitamin E requirements for growth and reproduction. Nebraska Agric. Exp. Station, 62nd Ann. Report: 81.

HERB, S. F., AND R. W. RIEMENSCHNEIDER 1953 Spectrophotometric micromethod for determining polyunsaturated fatty acids. Anal. Chem., *25:* 953.

HOVE, E. L. 1953 The toxicity of tri-o-cresyl phosphate for rats as related to dietary casein level, vitamin E and vitamin A. J. Nutrition, *51:* 609.

HOVE, E. L., D. H. COPELAND AND W. D. SALMON 1949 A fatal vitamin E deficiency characterized by massive lung hemorrhage and liver necrosis. Ibid., *39:* 397.

HOVE, E. L., AND J. O. HARDIN 1951 Effect of vitamins E, B_{12} and folacin on CCl_4 toxicity and protein utilization in rats. Proc. Soc. Exp. Biol. Med., *77:* 502.

HOVE, E. L., AND P. L. HARRIS 1947 Relative activity of the tocopherols in curing muscular dystrophy in rabbits. J. Nutrition, *33:* 95.

HOVE, E. L., AND Z. HOVE 1944 The chemical estimation of α-tocopherol and total tocopherol in mixtures of the β, α, and γ forms. J. Biol. Chem., *156:* 601.

LALOR, R. J., W. L. LEOSCHKE AND C. A. ELVEHJEM 1951 Yellow fat in the mink. J. Nutrition, *45:* 183.

LICHTMAN, S. S. 1949 Diseases of the liver, gallbladder and bile ducts. Lea and Febiger, Phila. p. 533.

MATET, A., J. MATET AND O. FRIDENSON 1949 Action du Dl, α-tocopherol sur la necrose hepatique du rat, provoquee par le Soja cru. Comp. rend. Soc. Biologie, *143:* 235.

OBEL, A. L. 1953 Studies on the morphology and etiology of so-called toxic liver dystrophy (Hepatosis Diaetetica) in swine. Acta pathologica et microbiologica Scand. Supplement, *94:* 1.

ROBINSON, K. L., AND W. E. COEY 1951 A brown discoloration of pig fat and vitamin E deficiency. Nature, *168:* 997.

SCHWARZ, K. 1944 Tocopherol als Leberschutzstoff. Z. physiol chemie, *281:* 109.

EFFECT OF AMINO ACID SUPPLEMENTS ON GROWTH AND FAT DEPOSITION IN THE LIVERS OF RATS FED POLISHED RICE [1]

A. E. HARPER, MARIE E. WINJE, D. A. BENTON AND C. A. ELVEHJEM

Department of Biochemistry, University of Wisconsin, Madison

(Received for publication December 20, 1954)

INTRODUCTION

It has been demonstrated that the relative proportions as well as the actual amounts of amino acids in the diet are important factors affecting the deposition of liver fat in rats fed low-protein diets containing choline (Singal et al., '53; Harper et al., '54a; Winje et al., '54). Since an accumulation of fat is one of the signs of the nutritional disease kwashiorkor, and since this disease is observed primarily among children consuming low-protein diets of plant origin, it was considered important, as other investigators have suggested (see Shils and Stewart, '54 for reference), to study the deposition of liver fat in rats fed low protein diets composed largely of cereals. The results of the initial phase of such an investigation, which are presented in this paper, indicate that the deposition of liver fat in rats fed rice diets containing adequate choline is distinctly affected by the balance of the dietary amino acids.

The results obtained by Pecora and Hundley ('51) and Pecora ('53), who studied the effects of amino acid supple-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the National Livestock and Meat Board, Chicago, Ill.; the Gelatin Research Society of America, Inc., New York City; and the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

ments on the growth of rats fed rice diets, were used as a basis for much of the present study on the deposition of liver fat and, as a consequence, many of their observations on growth response have been confirmed.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain weighing from 40 to 50 gm were divided into similar groups of 6 animals each and were maintained in individual cages with raised screen bottoms. The animals were fed ad libitum and were weighed weekly during the experimental periods of two weeks.

All of the diets contained 5% of corn oil, 4% of Salts 4 (Hegsted, Mills, Elvehjem and Hart, '41), and vitamins in milligrams per 100 gm of ration, as follows: thiamine hydrochloride 0.5, riboflavin 0.5, niacin 2.5, calcium pantothenate 2.0, pyridoxine 0.25, biotin 0.01, folic acid 0.02, vitamin B_{12} 0.002, inositol 10.0 and choline chloride 150. Halibut liver oil fortified with vitamins E and K was given orally each week (Harper et al., '54a). The remainder of the diet consisted of the percentages of the particular cereal, purified protein and amino acids indicated in the tables of results together with a small amount of sucrose to make up 100%. The percentages of L-lysine·HCl and DL-threonine were based on the observations of Pecora ('53) and the amounts of most of the other amino acid supplements were calculated to be equivalent to the quantities contained in 3% of casein (16% N-basis). The cereals, corn, wheat and polished rice, were commercial products and were ground finely.

After two weeks the rats were sacrificed for the determination of liver fat. Each animal was stunned and decapitated and the liver was removed and stored at —4°C. until the determinations were made. Fat was determined by ether extraction of the dried and ground liver (Hawk and Elvehjem, '53).

Nitrogen was determined by the Kjeldahl method using mercuric oxide as a catalyst.

RESULTS

Since the values for similar groups were quite consistent from experiment to experiment in this study, only representative results from a series of 7 experiments are presented in the tables. The values in all cases represent the average weekly gain and the average liver fat content for a group of 6 rats.

The results presented in table 1 show that rats fed a diet consisting of 90% of rice, which provided only 5.9% of protein, grew considerably better than rats fed an equivalent amount of corn, which provided 7.4% of protein, or a lesser amount of wheat, which provided 7.3% of protein. When each of these cereals was supplemented with casein and the diets were adjusted to contain approximately equivalent amounts of protein from the cereal and from the casein the rice diets proved superior at each protein level.

Only in the case of rats fed the 90% rice diet was there a marked accumulation of liver fat. The replacement of part of the rice with casein resulted in a substantial reduction in the deposition of liver fat. On the other hand, when part of the corn was replaced by 2.5% of casein a slight increase in the deposition of liver fat occurred. However, when the amount of casein in the corn diet was increased to 4.5% the deposition of liver fat was not increased.

The results presented in table 2 show the effects of supplements of various proteins and of the limiting amino acids lysine and threonine on growth and on the deposition of liver fat. No growth response was obtained with supplements of either 0.2% of L-lysine·HCl or 0.24% DL-threonine but when both of these amino acids were included in the rice diet in these amounts a substantial growth response occurred. When the lysine and threonine supplements were increased, to 0.4% and 0.5% respectively, the growth response was somewhat decreased. Although this decrease was relatively small, it occurred in 5 separate experiments. Each of the protein supplements listed in table 2 improved growth but there was some variation among them. The rate of gain

TABLE 1

Growth and liver fat deposition of rats fed various cereal diets

GROUP NO.	COMPOSITION OF DIET	PROTEIN CONTENT OF DIET	RATE OF GAIN	LIVER FAT	
				Dry wt.	Wet wt.
		%	*gm/wk.*	%	%
1	90% rice	5.9	8.8 ± 0.6 [1]	31.8 ± 2.6 [1]	10.8 ± 1.6 [1]
2	85% rice + 2.5% casein	7.7	23.7 ± 1.9	19.5 ± 1.6	5.6 ± 0.5
3	85% rice + 4.5% casein	9.5	33.0 ± 1.6	15.2 ± 0.7	4.4 ± 0.2
4	90% corn	7.4	2.2 ± 0.9	12.4 ± 1.8	3.4 ± 0.6
5	67% corn + 2.5% casein	7.7	9.8 ± 1.2	19.9 ± 3.5	6.0 ± 1.3
6	67% corn + 4.5% casein	9.5	19.5 ± 2.3	12.7 ± 0.8	3.5 ± 0.2
7	88% wheat	9.1	9.9 ± 1.2	11.8 ± 1.2	3.1 ± 0.4
8	71% wheat	7.3	4.6 ± 0.7	13.3 ± 1.1	3.6 ± 0.4
9	66% wheat + 2.5% casein	9.0	16.7 ± 1.1	14.0 ± 0.8	4.0 ± 0.1

[1] Values represent mean ± standard error of the mean for a group of 6 rats.

TABLE 2

Effect of protein and amino acid supplements on growth and liver fat deposition of rats fed polished rice diets

GROUP NO.	COMPOSITION OF DIET			SUPPLEMENT	RATE OF GAIN	LIVER FAT	
	Rice	L-lysine·HCl	DL-threonine			Dry wt.	Wet wt.
	%	%	%		*gm/wk.*	%	%
1	89	..	....	....	8.8 ± 0.7 [1]	28.0 ± 1.7 [1]	8.4 ± 0.6 [1]
2	89	0.2	....	....	8.5 ± 1.1	25.7 ± 2.9	7.8 ± 1.1
3	89	..	0.24	....	8.2 ± 0.8	31.9 ± 4.7	10.4 ± 2.0
4	89	0.2	0.24	....	24.6 ± 1.4	29.1 ± 2.2	8.9 ± 0.9
5	89	0.4	0.5	....	21.8 ± 2.7	15.2 ± 0.8	4.3 ± 0.2
6	89	..	..	1.5% glycine	6.8 ± 0.4	20.0 ± 1.2	5.4 ± 0.5
7	84	..	..	6.0% gelatin	24.0 ± 2.3	16.4 ± 0.9	4.6 ± 0.3
8	84	..	..	6.6% soy bean meal [2]	21.4 ± 1.1	20.8 ± 1.1	6.1 ± 0.2
9	84	..	..	3.2% pork [2]	28.6 ± 1.8	16.0 ± 0.6	4.6 ± 0.2
10	84	..	..	4.3% fish meal [2]	29.8 ± 1.2	16.7 ± 0.2	4.8 ± 0.1
11	84	..	..	3.3% casein [2]	32.0 ± 1.8	16.6 ± 0.9	4.8 ± 0.3
12	84	..	..	3.3% fibrin [2]	29.2 ± 0.8	16.4 ± 0.7	4.6 ± 0.3
13	84	..	..	3.7% egg albumin [2]	23.6 ± 1.0	18.6 ± 0.7	5.2 ± 0.2

[1] Values represent mean ± standard error of the mean for a group of 6 rats.

[2] Calculated to provide 3% of protein (N × 6.25).

obtained when the rice was supplemented with 3.0% protein as casein, pork, fish meal, or fibrin is equivalent to that obtained with a good purified diet over a two-week period.

Although the rate of gain of rats fed the rice diet supplemented with both 0.2% of L-lysine·HCl and 0.24% of DL-threonine was much greater than that of the controls, the deposition of liver fat was not decreased. In contrast, when the levels of L-lysine·HCl and DL-threonine were increased to 0.4% and 0.5%, respectively, the fat content of the liver was substantially decreased. The deposition of liver fat was also decreased in each case in which the rice diet was supplemented with protein. Soybean meal was the least effective of the supplements tested.

The results presented in table 3 were obtained in a group of three experiments designed to determine the effect on growth and liver fat deposition of supplements of what were calculated to be the limiting amino acids in the rice diets. It is evident from a comparison of the first 5 groups that an increase in the level of supplementary DL-threonine from 0.2% to 0.5% in the presence of 0.2% of L-lysine ·HCl produced no further growth response and very little reduction in the deposition of liver fat. In contrast, when the level of the L-lysine·HCl supplement was increased from 0.2% to 0.4% in the presence of 0.24% of DL-threonine a smaller growth response was obtained but there was a substantial reduction in the deposition of liver fat.

Since the calculations summarized by Flodin ('53) suggested that histidine, tryptophan and methionine should be the next most limiting amino acids after lysine and threonine, the effects of supplements of these amino acids were tested with the higher levels of lysine and threonine. Groups 6 to 9 are representative of two complete experiments in which it was observed that, although the deposition of liver fat was decreased in every case in which the higher levels of lysine and threonine were included in the diet, the addition of histidine, tryptophan and methionine in various combinations with 0.4% L-lysine·HCl and 0.5% DL-threonine, did not pre-

TABLE 3

Effect of amino acid supplements on growth and liver fat deposition of rats fed polished rice diets

GROUP NO.	COMPOSITION OF DIET			OTHER AMINO ACIDS	RATE OF GAIN	LIVER FAT	
	Rice	L-lysine·HCl	DL-threonine			Dry wt.	Wet wt.
	%	%	%		*gm/wk.*	%	%
1	89	..	...	....	8.0 ± 0.4 [1]	32.5 ± 3.7 [1]	10.9 ± 1.7 [1]
2	87	0.2	0.24	....	20.3 ± 1.5	29.9 ± 2.9	9.3 ± 1.1
3	87	0.4	0.5	....	16.6 ± 1.1	12.9 ± 0.9	3.7 ± 0.3
4	87	0.2	0.5	....	20.6 ± 0.7	26.5 ± 2.4	8.5 ± 0.9
5	87	0.4	0.24	....	18.9 ± 1.5	10.5 ± 0.4	2.9 ± 0.2
6	87	0.4	0.5	0.16% L-histidine·HCl	16.0 ± 1.0	14.0 ± 1.0	4.1 ± 0.3
7	87	0.4	0.5	0.05% DL-tryptophan	14.0 ± 1.5	12.9 ± 0.7	3.6 ± 0.2
8	87	0.4	0.5	0.1% DL-methionine	14.5 ± 0.9	15.0 ± 0.7	4.3 ± 0.2
9	87	0.4	0.5	hist. + trypt. + meth.[2]	16.5 ± 1.8	15.1 ± 0.8	4.3 ± 0.2
10	87	0.4	0.5	arg. + leuc. + isoleuc. + val. + phenylal.[2]	16.0 ± 1.7	12.6 ± 0.7	3.5 ± 0.2
11	87	0.2	0.24	essential amino acids ≅ 3.3% casein [2]	25.5 ± 1.0	27.8 ± 2.6	8.8 ± 0.9
12	87	0.4	0.5	essential amino acids ≅ 3.3% casein [2]	30.6 ± 1.7	14.9 ± 0.7	4.2 ± 0.2

[1] Values represent mean ± standard error of the mean for a group of 6 rats.

[2] Groups 11 and 12 received the following: L-arginine·HCl 0.16%, L-histidine·HCl 0.14%, DL-tryptophan 0.05%, DL-phenylalanine 0.2%, DL-methionine 0.1%, L-leucine 0.3%, DL-isoleucine 0.44%, DL-valine 0.46%. Amino acids included in diets 9 and 10 were also at these levels.

vent a decrease in the rate of growth. Similar results were obtained with a mixture of the other essential amino acids (group 10, table 3). Only when a mixture of all of the essential amino acids was included in the diet (groups 11 and 12, table 3) was a further growth response obtained and again only with the higher levels of lysine and threonine was there a substantial reduction in the deposition of liver fat.

DISCUSSION

The liver fat results are of interest both in themselves and as an extension of previous studies in which partial deficiencies of threonine and lysine have been shown to cause an increase in the deposition of liver fat (Singal et al., '53; Harper et al., '53a). In the present study, although threonine and several other essential amino acids were present in suboptimum amounts in the rice diet, the deficiency of lysine was evidently primarily responsible for the accumulation of liver fat because only when the level of this amino acid was increased to 0.4% of the diet was the deposition of liver fat decreased substantially (cf. groups 2 to 6, table 3). It is also interesting to note that soybean meal, which is known to be low in lysine, was the least effective of the protein supplements. A comparison of groups 11 and 12 (table 3), in which the slight growth depression caused by the additional lysine was overcome by supplementing the diet with all of the other essential amino acids, indicates that the reduction in the deposition of liver fat that occurred when the level of dietary lysine was increased could not have been a result of the decreased rate of growth.

Although the primary objective of this investigation was to study the deposition of liver fat rather than growth, nevertheless, the growth results confirm many of the observations of Pecora and Hundley ('51). As in their study, the rate of gain of rats fed rice diets was increased only when such diets were supplemented with both lysine and threonine, also, a further growth response was obtained only when a mixture of all of the other essential amino acids was provided. This

indicates, as they have concluded, that all of the other essential amino acids must be added concomitantly in order to improve further the growth of rats fed this rice diet. Throughout the present study the levels of lysine and of some of the other amino acids in the diet did not meet the stated requirement of the rat for growth, yet the rate of gain was considerably better than that reported by Pecora and Hundley who used much higher levels. Also the failure to overcome the growth-retarding action of the higher level of lysine with supplements of the other essential amino acids (groups 6–10, table 3 and several combinations not reported) indicate that very complex imbalances can be produced with ease using rice diets. It is thus possible that further investigation of the effects of different levels and balances of amino acids in rice diets may necessitate some modification of their conclusion.

The results of this study and that of Pecora and Hundley ('51) indicate, as they have pointed out, some of the pitfalls awaiting those who extrapolate from amino acid analysis, particularly amino acid analyses of plant products, to an estimate of the adequacy of a protein for growth. The method of determining protein "quality" as a function of the amino acid most limiting for growth (Block and Mitchell, '46–'47, Oser, '51) would be of very limited value without supporting evidence from animal experiments. This is further indicated by the work of Geiger et al. ('52) who have reported that certain of the amino acids in zein are only partially available to the rat.

The excellent growth obtained when the rice diet was supplemented with just 0.2% of L-lysine·HCl and 0.24% of DL-threonine, which provides the equivalent of only just over 6% of protein in the diet, is in marked contrast to the much poorer growth obtained when sucrose was used as the carbohydrate in diets containing 6% of casein supplemented with methionine, tryptophan and threonine (Harper et al., '54c). These results, together with those of other studies on the effect of the type of dietary carbohydrate on protein utilization (Harper and Katayama, '53; Womack et al., '53; Harper

et al., '53b; Marshall and Womack, '54; Dreisbach and Nassett, '54) demonstrate the importance of complex polysaccharides in the nutrition of rats (and probably of humans) consuming diets deficient in protein.

The results obtained in this study point up some of the problems that may be encountered when low-protein diets are supplemented with individual amino acids. Although the available information indicated that supplements of methionine, histidine and tryptophan should have been beneficial when included with threonine and lysine the inclusion of these amino acids either was without effect or was deleterious. Similar observations have been made by Sure ('54), Salmon ('54) and Winje et al. ('54) who used other proteins. Also, although growth was improved when the rice diet was supplemented with 0.2% L-lysine·HCl and 0.24% DL-threonine, this supplement did not prevent the accumulation of liver fat. On the other hand when the fatty infiltration was prevented by increasing the levels of lysine and threonine the growth response was somewhat less. These observations, together with those on the deleterious effects of excesses of certain of the essential amino acids (Graham et al., '50; Russell et al., '52; Hsu and Combs, '52; Harper et al., '54b) indicate that any program of amino acid supplementation of deficient diets should be preceded by a careful investigation.

The practical significance of the observation that fat accumulates in the livers of rats fed low-protein diets composed largely of rice remains to be determined. The rice diet used in this study, unlike the diets known to cause kwashiorkor, provided adequate quantities of the known essential dietary components other than amino acids. Therefore the effects of the amino acid deficiencies were not complicated by secondary deficiencies which might be encountered in most clinical studies. The interactions that may be expected when multiple deficiencies occur will require much further study. It is also apparent from a comparison of the results obtained with wheat, corn and rice diets (table 1) and from the study of Shils and Stewart ('54) that the use of different cereals

and particularly mixed diets will further complicate the picture.

SUMMARY

The effect of amino acid supplements on growth and on fat deposition in the livers of rats fed rice diets has been studied.

Fat accumulated to the extent of 8% to 10% (fresh wt.) in the livers of rats fed rice diets. The inclusion of 0.2% of L-lysine·HCl and 0.24% of DL-threonine in the rice diet did not prevent the accumulation of liver fat however the fat content of the liver was normal when 0.4% of L-lysine·HCl was included with either 0.24% or 0.5% of DL-threonine. Supplements of other amino acids were without effect on the deposition of liver fat.

In confirmation of the observation of Pecora and Hundley ('51) growth was improved only when both lysine and threonine were included in the rice diet and further improvement was obtained only when a mixture of all of the essential amino acids was included.

LITERATURE CITED

BLOCK, R. J., AND H. H. MITCHELL 1946–1947 The correlation of the amino-acid composition of proteins with their nutritive value. Nutrition Abst. Revs., *16:* 249.

DREISBACH, L., AND E. S. NASSET 1954 Absorption of carbohydrate and protein as affected by feeding cornstarch, banana or glucose. J. Nutrition, *53:* 523.

FLODIN, N. W. 1953 Amino acids and proteins, their place in human nutrition problems. J. Agric. Food Chem., *1:* 222.

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.

GRAHAM, C. E., S. W. HIER, H. K. WAITKOFF, S. M. SAPER, W. G. BIBLER AND E. I. PENTZ 1950 Studies on natural and racemic amino acids with rats. J. Biol. Chem., *185:* 97.

HARPER, A. E., AND M. C. KATAYAMA 1953 The influence of various carbohydrates on the utilization of low protein rations by the white rat. I. Comparison of sucrose and cornstarch in 9% casein ration. J. Nutrition, *49:* 261.

HARPER, A. E., D. A. BENTON, M. E. WINJE AND C. A. ELVEHJEM 1954a "Antilipotropic" effect of methionine in rats fed threonine-deficient diets containing choline. J. Biol. Chem., *209:* 159.

HARPER, A. E., D. A. BENTON, M. E. WINJE AND C. A. ELVEHJEM 1954b Leucine-isoleucine antagonism in the rat. Arch. Biochem. Biophys., *51:* 523.

HARPER, A. E., W. J. MONSON, D. A. BENTON AND C. A. ELVEHJEM 1953a The influence of protein and certain amino acids, particularly threonine, on the deposition of fat in the liver of the rat. J. Nutrition, *50:* 383.

HARPER, A. E., W. J. MONSON, D. A. ARATA, D. A. BENTON AND C. A. ELVEHJEM 1953b Influence of various carbohydrates on the utilization of low protein rations by the white rat. II. Comparision of several proteins and carbohydrates. Growth and liver fat. Ibid., *51:* 523.

HARPER, A. E., W. J. MONSON, D. A. BENTON, M. E. WINJE AND C. A. ELVEHJEM 1954c Factors other than choline which effect the deposition of liver fat. J. Biol. Chem., *206:* 151.

HAWK, E. A., AND C. A. ELVEHJEM 1953 The effects of vitamin B_{12} and B_{12r} on growth, kidney hemorrhage and liver fat in rats fed purified diets. J. Nutrition, *49:* 495.

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.

HSU, P.-T., AND G. F. COMBS 1952 Effect of vitamin B_{12} and amino acid imbalances on growth and levels of certain blood constituents in the chick. J. Nutrition, *47:* 73.

MARSHALL, M. W., AND M. WOMACK 1954 Influence of carbohydrate, nitrogen source and prior state of nutrition on nitrogen balance and liver composition in the adult rat. Ibid., *52:* 51.

OSER, B. L. 1951 Method for integrating essential amino acid content in the nutritional evaluation of protein. J. Am. Diet. Assn., *27:* 396.

PECORA, L. J. 1953 Effect of antibiotics on rats fed amino acid-supplemented rice diets. J. Nutrition, *49:* 621.

PECORA, L. J., AND J. M. HUNDLEY 1951 Nutritional improvement of white polished rice by the addition of lysine and threonine. Ibid., *44:* 101.

RUSSELL, W. C., M. W. TAYLOR AND J. M. HOGAN 1952 Effect of excess essential amino acids on growth of the white rat. Arch. Biochem. Biophys., *39:* 249.

SALMON, W. D. 1954 The tryptophan requirement of the rat as affected by niacin and level of dietary nitrogen. Ibid., *51:* 30.

SHILS, M. E., AND W. B. STEWART 1954 Development of portal fatty liver in rats on corn diets; response to lipotropic agents. Proc. Soc. Exp. Biol. Med., *85:* 298.

SINGAL, S. A., S. J. HAZAN, V. P. SYDENSTRICKER AND J. M. LITTLEJOHN 1953 The production of fatty livers in rats on threonine- and lysine-deficient diets. J. Biol. Chem., *200:* 867.

SURE, B. 1954 Relative nutritive values of proteins in whole wheat and whole rye and effect of amino acid supplements. J. Agr. Food Chem., *2:* 1108.

WINJE, M. E., A. E. HARPER, D. A. BENTON, R. E. BOLDT AND C. A. ELVEHJEM 1954 Effect of dietary amino acid balance on fat deposition in the livers of rats fed low protein diets. J. Nutrition, *54:* 155.

WOMACK, M., M. W. MARSHALL AND A. B. PARKS 1953 Some factors affecting nitrogen balance in the adult rat. Ibid., *51:* 117.

THE DOMESTIC CAT AS A LABORATORY ANIMAL FOR EXPERIMENTAL NUTRITION STUDIES

IV FOLIC ACID DEFICIENCY [1]

ALBERTO CARVALHO DA SILVA, REBECCA CARLOTTA DE ANGELIS,[2] MARIA APPARECIDA PONTES AND M. F. MANSUR GUÉRIOS

Department of Physiology,[3] Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brasil

(Received for publication December 5, 1954)

The requirements of mammals for folic acid have been studied in considerable detail in the rat, the pig and the monkey. The outstanding signs of deficiency are weight loss, anemia and leucopenia; diarrhea, skin and oral lesions, and fur depigmentation, are also described.

To produce a deficiency in monkeys (Waisman and Elvehjem, '43), foxes (Schaefer et al., '47) and mink (Schaefer et al., '46), it is sufficient to supply a well balanced purified ration without folic acid. In rats and pigs it is usually necessary to include sulfa drugs or anti-folic acid agents. Dogs are able to live on a purified ration without folic acid for as long as $4\frac{1}{2}$ years without signs of deficiency (Seeler and Silber, '45), but the vitamin may be necessary for complete recovery after niacin deficiency (Krehl et al., '46).

It is stated that the depleted rat (Asenjo, '48) and the pig (Cartwright and Wintrobe, '49) can be restored to the normal state by treatment with folic acid. On the other hand,

[1] This paper is based on data contained in a thesis presented in 1954 to the Faculty of Medicine of the University of S. Paulo, Brazil, by one of the authors (A.C.S.), in order to obtain the degree of "livre docente" on Physiology. The title of the thesis is as follows: Estudos Sobre A Carência De Ácido Fólico No Gato Doméstico.

[2] On a grant from Merck Institute For Therapeutic Research.

[3] Head, Professor Franklin A. de Moura Campos.

the fox (Schaefer et al., '48a) mink (Schaefer et al., '48b) and rhesus monkey (Cooperman et al., '46) require additional supplementation with liver or milk. This has been taken as evidence for the existence of an unknown anti-anemia factor for the monkey (Ruegamer et al., '48). A vitamin B_{12}-sparing action of large amounts of folic acid (0.5 mg daily) has been claimed (Smith and Elvehjem, '51).

Many substances with folic acid activity have been isolated or synthesized. One of them, the citrovorum factor (Sauberlich and Baumann, '48), attracted more attention in view of its potency in conteracting anti-folic acid agents and also because its formation by tissues is related to ascorbic acid (Nichol and Welch, '50). In animals not requiring vitamin C, folic and folinic acids (synthetic citrovorum factor) exhibit identical activity, providing that one considers that the synthetic folinic acid is a racemic product containing 50% of the active form (Broquist et al., '52). However, in scorbutic monkeys, the folic acid requirement is increased and folinic acid is 10 times more active than folic acid (May et al., '51).

In this paper we supply data in support of the conclusion that the domestic cat requires folic acid for normal growth and blood picture when sulfa drugs are included in purified rations. Recovery is obtained by supplying either folic or folinic acid and is improved by simultaneous administration of vitamin B_{12} or liver extract. Additional data on blood clotting time, plasma iron, blood volume, fecal fat, folic acid content of feces and urine are also presented.

EXPERIMENTAL

The detailed techniques used in working with cats have already been described (Carvalho da Silva, '50a). Cats two to three months old were placed in screen bottom cages and received ad libitum water and the following purified ration: casein,[4] 33; gelatin, 2; lard, 5; peanut oil, 5; salt mixture,[5] 5; sulfaguanidine or sulfathalidine, 0.6 to 2; and sucrose to

[4] Vitamin-free (Labco).

[5] Phillips and Hart, '35.

make 100%. When glycine was used (group II, table 1), it replaced an equivalent weight of sugar. This diet was supplemented with vitamins administered separately. One milligram each of thiamine, riboflavin, pyridoxine, vitamin K and para-aminobenzoic acid; 4 mg of calcium pantothenate; 4 to 10 mg of niacin; 0.01 mg of *d*-biotin; 30 mg of inositol and 300 mg of choline, were dissolved in 1 ml of water, this mixture emulsified in 2 ml of cod liver oil and the resulting product given by mouth to each animal three times a week. Once a week, 15 mg of alpha-tocopherol were added. It has been demonstrated that this ration, when supplemented with 0.5 mg of folic acid three times a week, promotes a satisfactory growth rate and blood picture and maintains the animal in a good state of health for at least 12 months (Carvalho da Silva, '50b).

Blood was obtained from the saphenous vein, using heparin as anticoagulant. Hemoglobin was determined with the Evelyn photoelectric colorimeter, filter 540. Hematocrit, mean corpuscular volume (M C V), mean corpuscular hemoglobin (M C Hb) and mean corpuscular hemoglobin concentration (M C Hb C) were calculated according to Wintrobe ('51). For reasons to be explained, only M C V will be presented. Blood clotting time was measured by placing 0.5 ml of blood drawn without anticoagulant, into an "hemolysis" tube, and tipping at intervals of a few seconds until there was no flow. Differential leucocyte counts were made using Leishmann's stain.

Blood volume determinations were performed with Evans blue (T-1824) using the hematocrit to calculate the total blood (Chinard, '51). The method was adapted as follows: 3 ml of blood were collected from one of the saphenous veins; the dye, dissolved in saline at the concentration of 5 mg per milliliter, was injected into the radial vein (approximately 1.5 mg of dye per kilogram of body weight). The exact amount of solution injected was measured by weighing the syringe with the needle before and after the injection, and correcting for the specific gravity of the solution, as evaluated with a picnometer. Six minutes after the end of the injection, 3 ml

of blood were collected from the other saphenous vein. After centrifugation, 0.4 ml of stained plasma were diluted to 6 ml. Unstained plasma was used to prepare the blank and the standard. The readings were made on the Evelyn photoelectric colorimeter, with filter 620.

Plasma iron was determined by the KSCN method, as standardized by Teixeira Mendes and Germek ('50); 2 ml of plasma were incubated with 1 ml of 6 N HCl, for 10 minutes, at room temperature, and 2 ml of 25% trichloroacetic acid added. After centrifugation, 1.7 ml of the liquid were transfered to a micro-Evelyn cuvette; 0.2 ml of 30% KSCN and 0.02 ml of conc. HNO_3 were added. The readings were made on the micro-Evelyn, with filter 540. Blanks of the solutions were carried in routine. "Liquaemin" was found to be the heparin with the lowest "blank" value.

Total fecal fat was determined by the method of Van De Kamer et al. ('49). After saponification with 30% KOH, acidification and extraction with petroleum ether, the extract was titrated with KOH in isobutanol, using a solution of thymol blue in isobutanol as indicator.

Folic acid was measured by microbial assay, with *S. faecalis*, and using the medium described by Roberts et al. ('46). The results were not affected by conjugase treatment (acetone extracted chicken pancreas), although the conjugase was very active, as tested with yeast extract.

To collect feces and urine, the animals were placed in screen bottom cages; under the cage was placed an enameled tray covered with a fine wet cloth, supported by a wire screen [6]. Prior to analysis, the samples were kept below 5°C., under toluene.

RESULTS AND DISCUSSION

The different techniques used to obtain folic acid deficiency in the cat and the hematological results observed, are presented in table 1. Glycine was tried (Group II) because there is

[6] It was recently observed in our laboratory that, whenever possible, high fat rations should be used to collect feces and urine, in order to avoid diarrhea.

TABLE 1

Hematological values of cats submitted to rations without folic acid under different experimental conditions

GROUP	NUMBER OF ANIMALS	EXPERIMENTAL CONDITIONS	DAYS ON EXPERIMENT		HEMATOLOGICAL DATA AT THE END OF THE EXPERIMENT [1]			
			Without sulfa drugs	With sulfa drugs	Hemoglobin	Red cells ($\times 10^6$/mm^3)	MCV	White cells ($\times 10^3$/mm^3)
					gm %			
Controls [2]	9	Purified ration without sulfa drugs and with folic acid	140–460	...	12.9	8.1	49	18.0
I	4	Without folic acid and without sulfa drugs	252 (165–393)	...	12.4 (10.8–14.8)	7.7 (5.9–9.2)	52 (42–64)	15.3 (10.0–20.4)
II	4	Same as group I, but with 5% glycine	150	...	11.7 (10.1–12.8)	6.2 (5.7–7.6)	59 (52–69)	16.1 (9.1–22.1)
III	14	Same as group I, but subjected to 2 or 3 periods of niacin deficiency	186 (110–246)	...	10.9 (8.4–12.2)	6.2 (4.5–8.4)	57 (44–70)	15.7 (6.2–30.2)
IV	8	Without folic acid and with sulfa drugs since the beginning of experiment	...	172 (129–196)	9.0 (6.0–12.4)	4.9 (3.6–7.2)	62 (49–74)	8.7 (3.5–13.6)
V	12	First period as in group I; second period as in group IV	147 (120–393)	85 (24–130)	8.4 (3.5–11.5)	4.4 (3.1–6.0)	60 (41–82)	5.6 (2.0–11.5)
VI	6	First period as in group III; second period as in group IV	226 (183–246)	59 (17–100)	7.0 (4.5–9.6)	4.3 (3.7–5.6)	52 (44–59)	5.6 (2.3–11.8)
VII	3	First period as in group I; second period as in group IV but with 22% casein	70	70	7.4 (5.2–9.6)	4.6 (3.1–6.0)	51 (45–62)	10.1 (9.2–11.5)

[1] Mean corpuscular hemoglobin (MCHb) and mean corpuscular hemoglobin concentration (MCHbC) are not included because MCHb follows mean corpuscular volume (MCV) in its variation. MCHbC is not affected.

[2] See Carvalho da Silva ('50a); values for adult males and females on purified ration were averaged.

experimental evidence that folic acid is utilized in glycine metabolism (Bessey et al., '53). This led us to consider the possibility that the glycine supplement might accelerate the exhaustion of tissue stores of folic acid. Niacin deficiencies were tried in view of the observation by Krehl et al. ('46).

Rations without sulfa drugs do not produce evident signs of deficiency (groups I, II and III, table 1). Some animals subjected to niacin depletion (group III), exhibit sub-normal values for hemoglobin, red cells and leucocytes and require folic acid for complete recovery after niacin treatment. However, the results are not consistent. When sulfa drugs are included from the beginning of experiment, the animals grow and exhibit normal blood picture during three to 5 months but finally lose weight and the blood values become subnormal. After one to three months of weight loss, most of these animals become anemic and leucopenic. Although they may stand a loss of weight of as much as 40% of their previous maximum in a fairly good state of health, they look depressed and treatment by folic acid is urgently needed when advanced anemia and leucopenia are present. If the animals are subjected to a preliminary period without sulfa drugs and without folic acid (groups V, VI and VII, table 1), the deficiency is obtained in a shorter time after the inclusion of sulfa drugs, and anemia and leucopenia are more regularly observed. The lowering of the casein content to 22% (group VII, table 1) shortens the experimental period but this technique is not advisable because, according to some of our unpublished results, this level of casein is low for the cat.

Ten adult animals were maintained on a purified ration with sulfa drugs and without folic acid for 130 days and subjected to frequent bleedings amounting to a total of 200 ml of blood per animal. Although regeneration of red cells and hemoglobin was slower by the end of the experiment, no consistent results could be obtained. This technique is troublesome and the results are difficult to interpret because the rate of blood regeneration was much slower on the completely purified ration than on stock diets.

Four cats, previously maintained on a ration without sulfa drugs and without folic acid, received 0.1 mg of aminopterin daily for 5 days. An acute deficiency was obtained characterized by leucopenia, hemoconcentration, weight loss, oral lesions with foul smell, and extensive intestinal hemorrhages. Treatment with 15 mg of folic acid plus 200 to 500 mg of ascorbic acid daily, initiated at the 5th day of the experiment, was effective in only one animal. The others died by the 7th or 8th day of experiment, although in one of them leucocyte counts were returning to normal.

From a study of table 1, it is evident that under these experimental conditions at least, the anemia has a tendency to macrocytosis. This is in agreement with published observations on chicks (Campbell et al., '45), rats (Kodicek and Carpenter, '50) and pigs (Cartwright et al., '50) although in this last-named species normocytosis was reported as the condition most frequently seen. In the monkey it is considered as macrocytic by some authors and normocytic by others (Day, '44).

Some of our animals were studied for blood clotting time. In 12 controls (purified ration without sulfa drugs and with folic acid) the average was 5 minutes (2 to 7); in 13 folic acid-deficient cats, the average was 9 minutes (3 to 25). In connection with these data, it should be mentioned that Day ('44) reports thrombocytopenia in the monkey, although in chicks the reduction of thrombocytes is of mild degree (Campbell et al., '45).

There is an evident rise in the level of plasma iron in folic acid-deficient cats. The average of 10 controls (purified ration without sulfa drugs and with folic acid) was 106 μg (50 to 200). In 16 deficient animals, the average was 208 μg (45 to 281). After treatment by folic acid, the values became normal before any response of hemoglobin and red cells was evident. An iron "load test" was performed on two deficient cats and two normal controls; 4 mg of iron (as ferrous sulfate) per kilogram of body weight and 200 mg of vitamin C were dissolved in 20 ml of water and given orally to fasting animals,

early in the morning. Blood samples were collected before 3, 6 and 9 hours after the "load" was given. In the deficient cats the values were high before the test (200 μg and 210 μg per 100 ml of plasma) and the same levels were maintained during the subsequent 9 hours. In the controls the values before the "load" were normal and there was a definite rise after the "load" was given. This is in agreement with the observations by Wills and Bilimoria ('32) who reported iron deposits in the livers of their monkeys.

The results on plasma and total blood volumes were as follows: (a) controls (7 cats on purified ration without sulfa drugs and with folic acid): plasma, 3.74%; total blood, 6.71%; (b) purified ration without folic acid and with sulfa drugs but without signs of deficiency (8 cats): plasma, 4.2%; total blood, 6.6%; (c) as in group b, but with a mild degree of anemia (7 cats): plasma, 4.2%, total blood, 6.6%; (d) deficient animals with pronounced anemia (3 cats): plasma, 4.1%; total blood, 6.1%. From these data it may be concluded that the blood volume is not subjected to variation at least until the animals become severely anemic. More data are necessary to decide whether there is a total blood reduction during the advanced anemia, but it is evident that there is no modification in plasma volume.

In leucopenic cats all types of white cells are reduced. The changes in differential counts during leucopenia are difficult to evaluate in view of the considerable variation exhibited by both normal and leucopenic animals. Panleucopenia with individual variations was also reported in the monkey (Day, '44) but in the rat there is a tendency to agranulocytosis (Kornberg et al., '43). Although there is no tendency to agranulocytosis in the leucopenic cats, the initial response to folic acid treatment is predominantly a granulopoiesis. The average values for 9 deficient animals were: Promyelocytes, 0.0%; myelocytes, 0.15%; metamyelocytes, 1.5%; mononuclears, 9.3%; polymorphonuclears, 28.0%; lymphocytes, 54.7%; monocytes, 4.35%; eosinophils, 2.0%. On 5 cats observed two days after treatment with folic acid, the averages were, pro-

myelocytes, 1.1%; myelocytes, 6.5%; metamyelocytes, 10.3%; mononuclears, 29.4%; polymorphonuclears, 34.1%; lymphocytes, 14.3%; monocytes, 3.6%; eosinophils, 0.5%.

As acid steatorrhea is a characteristic and specific feature of sprue in man (Spies, '47), the total fecal fat excretion of folic acid-deficient cats was determined in order to see whether from this point of view, this animal is comparable to man. The average values in three deficient animals for a three day collection period, was 0.66 gm daily; on 4 normal controls, on purified ration without sulfa drugs and with folic acid, the average was 0.77 gm daily.

The effect of sulfa drugs in precipitating deficiency is usually explained by the bacteriostatic effect on the intestinal flora (Daft and Sebrell, '45). Our results on fecal folic acid agree with this explanation; in 10 cats maintained on a purified ration without sulfa drugs and without folic acid, the average daily excretion of this vitamin was 23.5 μg; in 14 animals on the same ration but with sulfa drugs the average daily excretion was 8.5 μg. However, it is evident from the results in table 1, that intestinal synthesis is not sufficient to supply the optimum level, at least on the purified ration used. In fact, the animals maintained from the beginning on the ration with sulfa drugs and without folic acid developed deficiency after 6 months (group IV, table 1); but two and one-half months were sufficient when the animals were subjected to a preliminary period without sulfa drugs and without folic acid (group V, table 1). From these results one would expect cats on purified rations without sulfa drugs and without folic acid to excrete less folic acid in the urine than those receiving the same ration but with 0.5 mg of folic acid on alternate days. However the excretions were identical in both groups (2.2 mμg/ml of urine on 6 animals without folic acid and 2.3 mμg/ml of urine on 4 animals receiving 0.5 mg of folic acid on alternate days, providing that during the collection period, folic acid is not given).

These results cast some doubt on the adequacy of the folic acid content of the complete vitamin supplement used by us (0.5 mg every two days), although as will be shown later, a single dose of 2 mg produces an evident recovery in deficient animals. It is also reasonable to assume that the cat does not store folic acid in its tissues but excretes all of the excess in a very short time. On the other hand, from unpublished studies in our laboratory, it is possible to conclude that the folic acid level of tissues of cats maintained on rations without sulfa drugs is lower when folic acid is not included in the vitamin supplement; this is true at least for the adrenal, skeletal muscle, kidney, brain, cerebellum, testis, small intestine, heart, pancreas, spleen and skin. It is difficult to reconcile these results with the observation that sulfa drugs are necessary to produce the deficiency. A satisfactory explanation would be that the sulfa drugs act in some manner on tissue metabolism. The presence of sulfonamide in the blood and urine of cats receiving sulfaguanidine or sulfathalidine in the ration has been consistently demonstrated in our animals.

Response to treatment: When deficient cats are treated with adequate amounts of folic acid or folinic acid, they present a leucocyte response after 6 to 10 hours and a body weight response 6 to 10 days after the beginning of treatment. This delay is a constant finding whether low or high doses are used even when associated with vitamin B_{12} or liver extract. It was not observed in other vitamin deficiencies in the cat (thiamine, riboflavin, niacin, pyridoxine and pantothenic acid). Growth responses were obtained with single doses of 1 mg of folic acid and with two doses of 0.8 mg of folinic acid administered 24 hours apart. As lower doses were not tried, the minimum for weight response cannot be stated. However, the weight response obtained with single 1 mg doses was not complete and did not last for longer than a month. With two or more milligrams of folic acid, either in single or divided doses, longer lasting responses could be obtained.

The hematologic recoveries are summarized in table 2. Values for white cells and mean corpuscular volume (MCV)

are not included since there were no significant differences between the groups. Although data on reticulocytes would be very interesting, the authors were unable to stain them satisfactorily, even though different methods were used.

TABLE 2

Hematological recoveries of folic acid-deficient cats 30 days after the beginning of different types of treatment

GROUP	NUMBER OF ANIMALS	TREATMENT (SEE TEXT)	GAINS	
			Hemoglobin	Red cells ($\times 10^6$/mm^3)
			gm %	
I	2	1 mg of folic acid	— 1.50	— 1.70
II	2	2 mg of folic acid	+ 1.45	+ 1.32
III	3	5 mg of folic acid	+ 1.33	+ 1.72
IV	5	10 mg of folic acid or more	+ 1.16	+ 1.92
V	3	1.6 to 6.5 mg of folinic acid	— 1.56	— 0.24
VI	2	20 mg of folinic acid	+ 1.45	+ 1.71
VII	4	5 or more mg of folic acid, plus liver extract or vitamin B_{12} [1]	+ 3.75	+ 3.21
VIII	2	0.75 mg of folinic acid plus vitamin B_{12} [2]	+ 4.70	+ 2.38

[1] 20 μg of vitamin B_{12} daily during 5 days, or 0.5 ml of "Reticulogen Lilly" daily during 5 days.

[2] 20 μg of vitamin B_{12} daily during 5 days.

From table 2, it is evident that 1 mg of folic acid (single doses) or 1.6 to 6.5 mg of folinic acid [7] divided in two to 5 daily doses, did not promote hematologic recovery for red cells and hemoglobin. Larger amounts of folic or folinic acid were effective. However, when either vitamin B_{12} (20 μg daily during 5 days) or liver extract [8] were given together with folic or folinic acid, better recoveries were obtained.

[7] Leucovorin (Lederle) or folinic acid (Lilly).

[8] "Reticulogen Lilly", 0.5 ml during 5 days.

Some of the animals failed to exhibit a good response to folic acid or folic acid plus vitamin B_{12} and vitamin C. In this case, they usually died from intercurrent diseases, especially an acute pneumonia with extensive pleural empyema. In two of such animals however, a good weight response was obtained with powdered milk added to the ration and with a liver extract prepared by extracting raw minced beef liver with boiling water for 5 minutes and filtering through packed cheese cloth. Both supplements were effective after 10 to 15 days of treatment and the animals relapsed as soon as the treatments were interrupted, so that the effect of both could be demonstrated on the same animal. The amounts given daily were 20 gm of powdered milk and 50 ml of liver extract, corresponding to 30 gm of raw liver. It is possible that there is some relation between these observations and the monkey anti-anemia factor described by Smith and Elvehjem ('51).

When studying folic acid deficiency in the cat, one should carefully avoid confusing the deficiency with feline panleucopenia, particularly because anemia may also occur in this epidemic disease (Levaditti et al., '43). According to our experience, weight loss in infective leucopenia lasts for only 8 to 18 days (observations on 10 cats) and is followed either by death or spontaneous recovery. In any case, in the course of an experiment on folic acid deficiency it is advisable to maintain in the same room a group of animals on a completely purified ration, in order to exclude the possibility that the condition observed is an epidemic outbreak instead of a folic acid deficiency.

SUMMARY

Folic acid deficiency was obtained in the domestic cat by feeding a purified ration containing 0.6 to 2.0% of sulfaguanidine or sulfathalidine. The results were more uniform in animals subjected to a preliminary period during which both folic acid and sulfa drugs were not supplied. No fundamental difference was observed when the animals were subjected to

niacin deficiencies during this preliminary period. The deficiency signs were weight loss, anemia and leucopenia. The anemia had a tendency to macrocytosis. The leucopenia was characterized by a reduction in all types of leucocytes, but the response to folic acid, at least during the first two days, was predominantly granulopoietic. Blood clotting time was lengthened and plasma iron values were raised. Weight responses were obtained with single doses of 1 mg of folic acid or two doses of 0.8 mg of folinic acid. For hematologic recovery, single doses of 2 mg of folic acid were sufficient but if these were given together with vitamin B_{12} or liver extract, better results were obtained than with folic acid alone.

ACKNOWLEDGEMENTS

The authors take this opportunity to thank: *(a)* The Rockefeller Foundation for providing the vitamin-free casein and some of the vitamins needed for these studies; *(b)* Dr. Elmer Sevringhaus of Hoffmann La-Roche Inc., for *d*-biotin; *(c)* Elly Lilly and Company of Brasil Inc., for folinic acid and Reticulogen; *(d)* Laboratórios Lederle do Brasil, for leucovorin and aminopterin; *(e)* "Comissão de Pesquiza Científica da Universidade de S. Paulo", for its assistance. The valuable cooperation of Miss Maria Lucia Moreira and Mr. Alexandre José da Silva is gratefully acknowledged.

LITERATURE CITED

ASENJO, C. F. 1948 Pteroylglutamic acid requirement of the rat and a characteristic lesion observed in the spleen of the deficient animal. J. Nutrition, *36:* 601.

BESSEY, O. A., H. J. LOWE AND L. L. SALOMON 1953 Water soluble vitamins. Ann. Rev. Biochem., *23:* 545.

BROQUIST, H. P., J. A. BROCKMAN JR., M. J. FAHRENBACH, E. L. R. STOKSTAD AND T. H. JUKES 1952 Comparative biological activity of leucovorin and pteroylglutamic acid. J. Nutrition, *47:* 93.

CAMPBELL, C. J., M. M. MCCABE, R. A. BROWN AND A. D. EMMETT 1945 Crystalline vitamin B_c in relation to the cellular elements of chick blood. Amer. J. Physiol., *144:* 348.

CARTWRIGHT, G. E., AND M. M. WINTROBE 1949 Production of anemia in swine fed purified diets and sulfasuxidine. Proc. Soc. Exp. Biol. Med., *71:* 54.

CARTWRIGHT, G. E., J. G. PALMER, B. TATTING, H. ASHENBRUCKER AND M. M. WINTROBE 1950 Experimental production of nutritional macrocytic anemia in swine. III — Further studies on pteroylglutamic acid deficiency. J. Lab. Clin. Med., *36:* 675.

CARVALHO DA SILVA, A. 1950a The domestic cat as a laboratory animal for experimental nutrition studies. I — General considerations, care and feeding of the animals. Acta Physiol. Lat. Amer., *1:* 20.

———— 1950b The domestic cat as a laboratory animal for experimental nutrition studies. II — Comparative growth rate and hematology on stock and purified ration. Ibid., *1:* 26.

CHINARD, F. P. 1951 Estimation of plasma volume by dye dilution method. Methods in Medical Research, *4:* 38. The Year Book Publishers Inc. Chicago.

COOPERMAN, J. M., C. A. ELVEHJEM, K. B. MC CALL AND W. R. RUEGAMER 1946 "Folic acid" active compounds in the nutrition of the monkey. Proc. Soc. Exp. Biol. Med., *61:* 92.

DAFT, F. S., AND W. H. SEBRELL 1945 Sulfonamides and vitamin deficiencies. Vitamins and Hormones, *3:* 49.

DAY, P. L. 1944 The nutritional requirements of primates other than man. Ibid., *2:* 71.

KODICEK, E., AND K. J. CARPENTER 1950 Experimental anemias in the rat. I — Macrocytic anemia in chronic pteroylglutamic acid deficiency and after splenectomy in Bartonella muris infection. Blood, *5:* 522.

KORNBERG, A., F. S. DAFT AND W. H. SEBRELL 1943 Production and treatment of granulocytopenia and anemia in rats fed sulfonamides in purified diets. Science, *98:* 20.

KREHL, W. A., N. TORBET, J. DE LA HUERGA AND C. A. ELVEHJEM 1946 Relation of synthetic folic acid to niacin deficiency in dogs. Arch. Biochem., *11:* 363.

LEVADITTI C., P. LEPINE AND J. VERGE 1943 Les ultravirus des maladies animales. Librairie Maloine, Paris.

MAY, C. D., R. D. SUNDBERG, F. SCHAAR, C. U. LOWE AND R. J. SALMON 1951 Experimental nutritional megaloblastic anemia; relation of ascorbic acid and pteroylglutamic acid. I — Nutritional data and manifestations of animals. Am. J. Dis. Child., *82:* 282.

NICHOL, C. A., AND A. D. WELCH 1950 Synthesis of citrovorum factor from folic acid by liver slices; augmentation by ascorbic acid. Proc. Soc. Exp. Biol. Med., *74:* 52.

PHILLIPS, R. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

ROBERTS, E. C., AND E. E. SNELL 1946 An improved medium for microbiological assays with Lactobacillus casei. Ibid., *163:* 499.

RUEGAMER, W. R., E. M. SPORN, U. D. REGISTER AND C. A. ELVEHJEM 1948 Distribution and fractionation of the monkey anti-anemia factor. J. Nutrition, *36:* 405.

SAUBERLICH, H. E., AND C. A. BAUMANN 1948 A factor required for the growth of Leuconostoc citrovorum. J. Biol. Chem., *176:* 165.

SCHAEFER, A. E., C. K. WHITEHAIR AND C. A. ELVEHJEM 1946 Purified rations and the importance of folic acid in mink nutrition. Proc. Soc. Exp. Biol. Med., *62:* 169.

———— 1947 Purified rations and the requirement of folic acid for foxes. Arch. Biochem., *12:* 349.

———— 1948a Unidentified factors for growth and hemoglobin production in foxes. J. Nutrition, *35:* 147.

SCHAEFER, A. E., S. B. TOVE, C. K. WHITEHAIR AND C. A. ELVEHJEM 1948b The requirements of unidentified factors for mink. Ibid., *35:* 157.

SEELER, A. O., AND R. H. SILBER 1945 Studies on the prolonged maintenance of adult dogs on purified diets. Ibid., *30:* 111.

SMITH, S. C., AND C. A. ELVEHJEM 1951 An interrelation of pteroylglutamic acid vitamin B_{12} and the monkey anti-anemia factor. Ibid., *45:* 47.

SPIES, T. D. 1947 Experiences with folic acid. The Year Book Publishers Inc. Chicago.

TEIXEIRA MENDES, F., AND O. A. GERMEK 1950 Determinação quantitativa do ferro plasmático. Hospital, *37:* 129.

VAN DE KAMER, J. H., H. B. BOKKEL HUININK AND H. A. WEYERS 1949 Rapid method for the determination of fat in feces. J. Biol. Chem., *177:* 347.

WAISMAN, H. A., AND C. A. ELVEHJEM 1943 The role of biotin and "folic acid" in the nutrition of the rhesus monkey. J. Nutrition, *26:* 361.

WILLS, L., AND H. S. BILIMORIA 1932 Studies in pernicious anemia of pregnancy. Part V: Production of macrocytic anemia in monkeys by deficient feeding. Ind. J. Med. Res., *20:* 391.

WINTROBE, M. M. 1951 Clinical Hematology. Lea and Febiger, Philadelphia.

NUTRITIONAL STUDIES WITH THE GUINEA PIG

III. CHOLINE

MARY ELIZABETH REID

Laboratory of Biochemistry and Nutrition, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland

(Received for publication December 13, 1954)

Because of various difficulties which in the past have been encountered in obtaining a satisfactory growth rate of guinea pigs on purified diets, the necessity of a dietary supply of choline has only recently been shown (Reid, '54). The present study is concerned (1) with an extension of findings on choline deficiency previously reported, (2) with a determination of the dietary choline requirement, and (3) with an investigation of the ability of related compounds to serve as substitutes for choline.

METHODS

Hartley strain guinea pigs two to 4 days of age, ranging in weight from 90 to 115 gm, were used. Equal numbers of males and females were divided according to weight into uniform groups of 8 and were placed in individual screen-bottom cages and fed a semi-synthetic diet (Reid and Briggs, '53) which contained 30% vitamin-free casein, 7.3% fat, 4 types of carbohydrates, 6% salts and liberal amounts of the known vitamins except choline. A detailed account of the care of the animals was published previously (Reid and Briggs, '53). In setting up an experiment extra animals were included for replacement of losses which might occur during the first week as a result of diarrhea or mortality for one reason or another. The surviving animals were kept on their respective diets for

a period of 6 weeks. If blood studies were to be made, the samples were taken during the last week. At the end of the 6th week the animals were sacrificed and the internal organs were examined for gross pathology and samples of tissues were preserved for histological study. In some experiments the organs were weighed.

RESULTS

Choline deficiency experiments

Guinea pigs fed the diet lacking choline showed a marked retardation in growth by the end of the second week and by the end of the third week a few of them had succumbed. There was some difference in the rate of onset of the deficiency in different experiments for which no certain explanation has been found. In some tests not more than one or two of the 8 animals originally in the group were still alive by the end of the 4th week, whereas in others about half of the animals were alive at this time and some continued to live to the end of the 6-week experimental period. One experiment was continued as long as any of the animals lived. The last one died on the 67th day. The results of three experiments with no addition of choline are summarized in table 1.

Loss of appetite, roughness of fur, thinning of fur of some animals, muscular weakness, and a slight reddening of the paws developed as the deficiency progressed. At autopsy, a clear fluid was occasionally found in the body cavities, small subcutaneous and adrenal hemorrhages were frequently observed, also an increase in size of the adrenals with a tendency to thickening of the cortex. The kidneys tended to be pale, somewhat increased in size in relation to body weight; some had a slightly scarred surface. No acute hemorrhages were found. Only an occasional animal showed fatty infiltration of the liver. In a few of the animals the bone marrow was somewhat aplastic and the thyroids of two of those examined showed less colloid than normal.[1] Blood studies were made

[1] Study made by Dr. G. L. Fite, Laboratory of Pathology and Histochemistry, National Institutes of Health.

in some of the deficient animals; in all instances there was a reduction in the number of red cells and in hemoglobin but no definite alteration in the total number of leucocytes and polymorpho-nuclears (table 2). Failure in clotting of the blood was observed in a few of the animals at autopsy.

TABLE 1

Effect of different levels of dietary choline[1] *on growth and survival*

CHOLINE CHLORIDE	NO. OF ANIMALS	NO. OF SURVIVORS	AVERAGE WTS. AT SUCCESSIVE PERIODS (WKS.)		
			2	4	6
gm/kg			*gm*	*gm*	*gm*
none	24	6	124	136	156 ± 3[2]
0.2	8	5	143	181	219 ± 9
0.4	32	27	145	206	248 ± 4
0.5	32	28	147	211	273 ± 8
0.6	8	7	154	230	278 ± 20
0.8	16	15	167	244	299 ± 13
1.0	8	8	162	241	325 ± 15
1.2	8	8	169	255	310 ± 15
1.5	8	8	169	247	330 ± 26
2.0	56	55	155	242	322 ± 4
3.0	8	8	151	242	336 ± 14
5.0	8	8	144	235	326 ± 10

[1] Increases in the choline content of the diet above the control level (2.0 gm/kg) were made at the expense of starch. Decreases in the choline level were made by substitution of cerelose.

[2] Standard error determined by method of Mantel ('51).

TABLE 2

Effect of dietary choline on the blood constituents
(Animals on diets 5 to 6 weeks)

CHOLINE HCl	NO. OF ANIMALS	WEIGHT	ERYTHRO-CYTES	HEMATOCRIT	HEMOGLOBIN	TOTAL LEUCOCYTES	POLYMORPHO-NUCLEARS
gm/kg		*gm*	*10^6/ml*	*%*	*gm/100 ml*		
0	5	163	4.06 ± 0.75	31.3 ± 3.7	9.2 ± 1.1	5180 ± 1450	2860 ± 1120
0.2	7	184	...	34.1 ± 2.1	12.5 ± 0.7	9014 ± 1560	5364 ± 1080
0.5	7	257	5.20 ± 0.16	40.0 ± 0.66	13.6 ± 0.3	5629 ± 864	1743 ± 314
0.8	7	247	...	37.0 ± 0.7	13.3 ± 0.3	6807 ± 1536	3314 ± 886
1.5	8	255	...	41.4 ± 0.9	15.0 ± 0.5	5588 ± 1094	2706 ± 737
2.0	8	304	6.19 ± 0.3	42.4 ± 0.8	14.6 ± 0.3	5344 ± 731	2050 ± 387

Effect of varying levels of dietary choline chloride

The average values of weight and survival at different levels of choline chloride are shown in table 1. With the addition of only 0.2 gm/kg of diet growth was greatly improved and 5 of the 8 animals survived to the end of the 6-week experimental period. With each increase in dietary choline up to 1.0 gm/kg there was increase in growth, but particularly there was an increase in survival. With levels ranging from 1.0 to 5 gm/kg there was 100% survival. Maximum growth was obtained with levels of 1.0 to 1.5 gm/kg.

Some (about 20%) of the deaths which occurred in animals receiving 0.4 to 0.6 gm/kg of choline chloride in the diet were sudden. Appetite had been good and the animals had been gaining slowly but steadily. However, they had shown definite evidence of muscular weakness, particularly in the hind quarters. The ears and feet tended to be pale, and blood studies showed an anemic condition (table 2). One of the animals which died under observation was autopsied immediately and a large thrombus was found in the heart. The cause of the sudden death in the other animals was not ascertained but in most of them an unusually large amount of blood was found in the heart. Histological studies [2] of 16 animals with the chronic type of deficiency showed no lesions in the kidneys or adrenals. The livers of three of them showed a small amount of fat and only one showed a considerable deposition of fat. The hearts of only two of the 16 animals showed pathological changes. The effect on the blood of variations in the content of dietary choline is shown in table 2. The red cell picture was affected particularly. As the dietary choline approached the optimum content, the number of red cells, the hematocrit, and the hemoglobin all increased.

[2] Study made by Dr. R. T. Habermann, Laboratory of Pathology and Histochemistry, National Institutes of Health.

Value of compounds related to choline

Studies were also made to determine the value of other compounds as substitutes for choline. In different experiments amounts of the compounds employed were usually either (1) equal by weight to the level of dietary choline chloride used for the control group, (2) equal to the choline chloride on a molar basis, (3) equivalent as to methyl groups,[3] or (4) equivalent as to the aminoethanol moiety. The quantitative results are shown in table 3.

With 0.6 gm/kg of methionine in a diet lacking choline, growth was very poor and only one of the 8 animals survived to the end of the experimental period. With 1.63 gm/kg of methionine, the level required to yield methyl groups equal to the amount supplied by 0.5 gm/kg of choline chloride,[3] growth of some of the animals appeared to be slightly improved and there were three survivors. Addition of methionine in an amount (6.54 gm/kg) providing methyl groups equal to the amount found in 2.0 gm/kg of choline chloride resulted in no further improvement. With 0.5 gm/kg of choline chloride in addition to 1.63 gm/kg of methionine, growth and survival were greatly improved and were better than without the methionine but not equal to the results obtained with 1.0 gm/kg of choline chloride. The addition of 1.0 gm/kg of methionine to the diet containing 2.0 gm/kg of choline chloride resulted in no improvement in the results obtained without the added methionine.

Results obtained with betaine used as a substitute for choline were much like those described for methionine. With 0.6 gm/kg of betaine and no choline, growth was poor and there were no survivors. Both growth and survival appeared to be slightly improved by increasing the betaine so as to supply methyl groups equal to that in 2.0 gm/kg of choline chloride. Marked improvement in growth and survival re-

[3] The *total* methyl content of choline was used in computing the amounts of the substitution compounds employed. There is as yet no evidence for or against the postulate that in the guinea pig only one of the methyl groups of choline is used in transmethylation processes.

TABLE 3

Effect of compounds used as substitutes for, or as supplements to, choline

CHOLINE HCl	SUPPLEMENT		NO. OF SURVIVORS / TOTAL NO. USED	AVERAGE WTS. AT SUCCESSIVE PERIODS (WKS.)		
	Kind	*gm/kg*		2	4	6
gm/kg				*gm*	*gm*	*gm*
none	none		6/24	124	136	156
none	methionine	0.6	1/8	130	139	137
none	methionine	1.63	3/8	127	141	150
none	methionine	6.54	1/8	121	140	152
0.4	methionine	1.39	8/8	139	196	257
0.4	methionine	6.92	8/8	131	180	236
0.5	methionine	1.63	7/8	142	219	297
0.6	methionine	0.6	8/8	158	236	291
2.0	methionine	1.0	8/8	146	232	321
none	betaine	0.6	0/8	130	129	...
none	betaine	1.68	4/8	134	150	180
0.4	betaine	0.34	7/8	134	202	270
0.4	betaine	1.68	8/8	131	190	258
0.5	betaine	0.5	8/8	149	207	271
none	aminoethanol	0.75	5/8	121	135	154
none	aminoethanol	1.35	3/8	133	144	184
0.5	aminoethanol	0.75	8/8	135	210	281
none	N-methyl aminoethanol	3.22	2/8	140	150	...
none	dimethyl aminoethanol	0.55	8/8	142	228	310
none	dimethyl aminoethanol	1.91	8/8	151	253	337
0.4	dimethyl aminoethanol	0.48	8/8	153	229	320
0.5	dimethyl aminoethanol	0.55	8/8	150	236	320
0.5	dimethyl aminoethanol	0.5	8/8	154	231	292
none	cystine	5.95	4/8	126	149	160
none	homocystine	1.93	3/8	124	146	169
0.5	homocystine	0.5	7/8	152	213	275
none	methylglycine	3.83	3/8	127	155	180
none	dimethylglycine	2.21	7/8	129	153	185
none	sodium formate	3.37	5/8	129	143	165
0.5	stachydrine HCl	0.5	8/8	156	204	260
0.5	vitamin B_{12}	0.4 mg/kg	6/8	139	212	268
0.6	vitamin B_{12}	0.4 mg/kg	8/8	153	223	278
0.4	vitamin B_{12}	0	8/8	137	190	244

sulted from supplying suboptimum amounts (equimolar) of choline chloride (0.4 gm/kg) and betaine (0.34 gm/kg). The average weight at the end of the experimental period was greater than without the betaine but not as good as with double the amount of choline chloride. Addition of a relatively large amount of betaine (1.68 gm/kg) to a low choline (0.4 gm/kg) diet appeared to yield poorer growth than was obtained with the smaller amount (0.34 gm/kg) of betaine. Stachydrine HCl, which is closely related to betaine HCl, was used as a supplement to 0.5 gm/kg of choline. The results were similar to those obtained with betaine.

Aminoethanol was ineffective as a substitute for choline when fed at levels of 0.75 gm/kg and 1.35 gm/kg. Also, at the 0.75 gm/kg level it had no supplementary value in a diet containing 0.5 gm/kg of choline chloride (table 3). The general picture at autopsy was essentially the same as that observed in the unsupplemented animals.

N-methylaminoethanol, when fed at a level of 3.22 gm/kg, did not promote growth and mortality was high. Marked abnormalities were produced in the internal organs. Many of the animals showed hemorrhages in the gastrointestinal tract and most of them showed enlargement of the liver, kidneys, adrenals, and heart. The average weights of the organs per 100 gm body weight and the standard errors are shown in table 4 and are to be contrasted with those of the control group (2.0 gm/kg choline chloride). The livers had a peculiar stiff and inflexible character.

Dimethylaminoethanol proved to be an excellent substitute for choline. With 0.55 gm/kg of this compound, growth was similar to the results obtained with larger amounts of choline chloride (table 1). With 1.91 gm/kg, the equivalent to 2.0 gm/kg of choline chloride on a methyl basis, growth was equal to that obtained with the choline chloride. It also appeared to be equal in value to choline chloride when used as a supplement to an inadequate supply of the latter compound. Similarity in the effect of dimethylaminoethanol and choline chloride was shown at autopsy in the appearance

and weights of the internal organs. Per unit of body weight, the average weights of the livers, kidneys, adrenals, and hearts of the two groups were almost identical (table 4).

L-cystine and DL-homocystine were only slightly beneficial in a diet lacking choline. In general, the deficiency symptoms observed in the animals receiving cystine or homocystine in a diet lacking choline were somewhat more pronounced than those seen in the unsupplemented animals, possibly due to the longer survival time. With homocystine (1.93 gm/kg) employed as a substitute for choline the kidneys tended to

TABLE 4

Effect of different compounds[1] *on organ weights per unit body weight*[2]

(8 animals per group)

COMPOUND	AMOUNT	LIVER	KIDNEYS	ADRENALS	HEART
	gm/kg	*gm/100 gm*	*gm/100 gm*	*mg/100 gm*	*mg/100 gm*
No supplement	0	4.94 ± 0.80	1.28 ± 0.16	123 ± 14	406 ± 52
Choline chloride	2.0	5.93 ± 0.31	0.94 ± 0.05	48 ± 1.2	317 ± 14
Aminoethanol	0.75	5.48 ± 0.60	1.00 ± 0.06	102 ± 9	392 ± 8
N-methylaminoethanol	3.22	8.73 ± 1.48	1.71 ± 0.20	92 ± 4	571 ± 72
Dimethylaminoethanol	1.91	5.81 ± 0.54	0.98 ± 0.06	50 ± 2.6	354 ± 27

[1] Insignificant variations in organ weights in relation to body weight were found with betaine, methionine, cystine, homocystine, methylglycine, dimethylglycine, and formate.

[2] The determinations were made on the 42nd day except in those animals which succumbed earlier. The latter data were also included in the table.

be pale with a faded structure and the adrenals were hemorrhagic. Homocystine (0.5 gm/kg), when used as a supplement with 0.5 gm/kg of choline chloride, did not aggravate the effect of the inadequate choline supply.

In a test with methylglycine (3.83 gm/kg) as a substitute for choline, growth was poor and only three survivors remained at the end of the experimental period. With dimethylglycine (2.21 gm/kg), growth was also poor but there were 7 survivors. At autopsy, all of the dimethylglycine animals showed subcutaneous hemorrhages and most of them also had hemorrhages of the gastrointestinal tract; in one animal it was massive. The kidneys and the pancreas tended to be pale and some of the

animals had small hemorrhages in the adrenals. Sodium formate (3.37 gm/kg) was an ineffective substitute for choline. Insignificant variations in organ weights were found with these different compounds in relation to body weights as compared to those of the control animals.

The addition of large amounts of vitamin B_{12} (0.4 mg/kg of diet vs. 0.04 mg/kg in the regular purified diet) to a diet containing suboptimal amounts of choline (0.05 and 0.06 gm/kg) produced no improvement in growth. Omission of vitamin B_{12} from a low choline diet (0.4 gm/kg) caused no poorer growth than was obtained in tests with the vitamin present. It is possible that the 30% casein in the diet contained an ample amount of vitamin B_{12}.

TABLE 5

Effect of a dietary supply of both moieties of the choline molecule

COMPOUND ADDED TO DIET LACKING CHOLINE		AVERAGE WEIGHTS AT WEEKLY PERIODS					NO. OF SURVIVORS / TOTAL NO. USED
		2	3	4	5	6	
	gm/kg	*gm*	*gm*	*gm*	*gm*	*gm*	
None		124	130	143	155	158	2/8
Choline chloride	2.0	146	183	228	283	327	8/8
DL-Methionine	6.92	121	134	140	149	152	1/8
Methylaminoethanol	3.22	140	149	158	151	150	0/8
DL-Methionine + methylaminoethanol	4.00 1.36	147	188	237	288	329	8/8

Ability of the guinea pig to utilize methionine if the alcohol moiety of the choline molecule is supplied

A study was made to determine if the guinea pig can utilize methyl groups from methionine for the production of choline if the alcohol moiety of the choline molecule is also supplied. Monomethylaminoethanol was added in an amount sufficient to furnish aminoalcohol groups equal to those in two grams of choline chloride and methionine was added in an amount sufficient to supply the necessary methyl groups. The results are shown in table 5. In agreement with the previously described results, it was found that each of the

two compounds when fed alone in a choline-deficient diet resulted in no better growth or survival than was obtained with the deficient diet containing neither of these supplements. When the two compounds were fed together, growth equaled that obtained with 2.0 gm/kg of choline chloride.

DISCUSSION

It has been postulated (Cornatzer, '54) that choline deficiency cannot be produced in the guinea pig by dietary means because of the lack of, or weak development of, a choline oxidase system (Bernheim and Bernheim, '33; Handler and Bernheim, '42; Dubnoff, '49a, b; Wells, '54; Kensler and Langemann, '54). Handler ('49) had previously suggested that because of the lack of this enzyme the turnover of choline is so slow as to make the daily requirement of the guinea pig less than that of the rat. The results of the present experiments show, however, that the young guinea pig has a definite dietary requirement for choline not only for growth but for survival. In fact, under dietary conditions which are considered to be near the optimum for each species, it appears that choline is needed in about the same proportion as in the rat (Griffith, '54) and in the rabbit (Hove, Copeland and Salmon, '54). Although the requirement of older guinea pigs (half to full-grown) has not yet been definitely established, there is a report (Casselman and Williams, '54) that the addition of choline to a diet (presumably containing appreciable amounts of choline) stimulated the growth of pigs whose average starting weight was 550 gm.

The manner in which the guinea pig responded to the various compounds employed as substitutes for choline was strikingly different in several respects from that known for the rat. Neither betaine nor methionine if used alone substituted for choline but, if a moderate amount of either of these compounds was furnished in the presence of an inadequate supply of choline, an additive effect was observed although the response tended to be less than if an equivalent amount of

choline chloride had been supplied. In this respect the guinea pig is like the chick (McKittrick, '47, '48).

Although, in the rat, aminoethanol has been shown to serve as a precursor for the biological synthesis of choline (Stetten, '41), it appeared to have little, if any, value to the guinea pig either as a substitute for dietary choline or as a supplement to it when added to a 30% casein diet. The increased phospholipid turnover shown to occur with aminoethanol in the rat (Platt and Porter, '47; Artom and Cornatzer, '48; Pilgeram et al., '53) possibly occurs at a much reduced rate in the guinea pig, if to any extent at all, a response probably related to the slight tendency of the guinea pig to accumulate liver fat in the absence of dietary choline.

Monomethylaminoethanol was also unsatisfactory as a substitute for choline when added to the 30% casein ration. However, if dietary methyl groups were supplied by the addition of methionine, the animals did as well as with an equivalent amount of choline chloride. This result is in agreement with the findings of Jukes, Oleson and Dornbush ('45) in the chick. The fact that no benefit resulted from the addition of 1.0 gm/kg of methionine to the standard diet containing 2.0 gm/kg of choline chloride indicates that the methionine contained in the 30% casein is sufficient to satisfy the requirement of methionine as such in the guinea pig. Methionine was beneficial only when there was insufficient dietary choline and then only when the missing alcohol moiety was also supplied. On the other hand, the amount of methionine supplied by the 30% casein in the diet was not sufficient in a diet lacking choline to allow for utilization of methylaminoethanol with its requirement of two additional methyl groups. Further experiments with aminoethanol supplemented with methionine or betaine are now under way. Of interest in connection with these aminoethanol studies are the recently reported results of Pilgeram and Greenberg ('54) with liver slices in which they observed a lack of capacity of the guinea pig to form lecithin from free aminoethanol.

Among the many compounds tested, dimethylaminoethanol was the only one which was effective when used alone as a supplement to a diet lacking choline and it appeared to have a potency equal to that of choline chloride. None of the other compounds studied showed the striking parallelism as to growth, survival, general external and internal appearance of the animals, and organ weights with those obtained with choline chloride. These results, and those with monomethylaminoethanol supplemented with methionine, suggest that furnishing an alcohol moiety for the choline molecule is just as important for the guinea pig as is the supplying of methyl groups. The promotion of growth with dimethylaminoethanol in choline-deficient diets appears to be relatively greater in the guinea pig than in the rat (du Vigneaud et al., '46) or in the chick (Jukes and Oleson, '45). It would appear that in the guinea pig as in the rat and chick (du Vigneaud et al., '46; Jukes, '47) this compound probably acts as a methyl acceptor with the additional methyl groups presumably being furnished by the methionine contained in the casein. This would consequently suggest that with the type of diet herein employed the methionine content of the casein (about 3.4%) is considerably more than adequate to supply the requirement of the guinea pig for methionine as such.

The pathological effects of lack of choline in the diet are, in several of their major aspects, quite different from those found in the rat. Neither marked fatty infiltration of the liver (Handler, '49) nor kidney hemorrhages have been found. Disturbances in the red blood cell picture and muscular weakness appeared to be the most outstanding defects in the guinea pig. If a diet markedly higher in fat had been employed such as that used by Hove and Copeland ('54) in their study of muscular dystrophy in the rabbit, muscular defects in the guinea pig might have been more pronounced. The immediate cause of death in either the acute or the chronic deficiency in the present experiments was not determined. Handler ('49) had also found that pigs on diets low in choline died suddenly after periods of reasonable weight gain

and adequate food intake. Sudden deaths were not observed either in his animals maintained on the stock diet or in the animals maintained on the purified control diets of the present experiments.

Much more information could be gained from other studies with varying amounts of dietary fat and with choline substitution compounds used in different combinations. However, some of the questions pertaining to choline metabolism in the guinea pig may probably be answered only by the use of labeled compounds.

SUMMARY

Omission of choline from the diet of the young guinea pig resulted in severe retardation of growth, weakness of muscles, reduction in number of red blood cells, lower hematocrit and hemoglobin values, small subcutaneous and adrenal hemorrhages, paleness of the kidneys but no severe kidney hemorrhages, and no marked fatty infiltration of livers. Survival time varied from 21 to 67 days.

A chronic state of deficiency characterized by somewhat retarded growth, anemia, and marked weakness of muscles was produced by the inclusion of small amounts of choline in the diet.

From 1.0 to 1.5 gm of choline chloride per kilogram of diet was sufficient to afford 100% survival and maximum growth.

Neither betaine nor methionine produced any benefit as substitutes for choline but had a supplementary value when supplied with a suboptimal level of choline. However, in this instance they appeared to have somewhat less growth-promoting value than an equivalent amount of choline chloride.

Dimethylaminoethanol was the only single compound found to be equal in effect to choline HCl when fed on a molar basis.

Neither methylaminoethanol nor methionine improved growth or survival when used as supplements with a choline-deficient diet but when fed together in suitable amounts were as satisfactory as a corresponding level of choline chloride.

The guinea pig apparently requires a dietary supply of both moieties of the choline molecule to cover its metabolic requirements.

ACKNOWLEDGMENTS

The author wishes to express appreciation to Dr. G. M. Briggs and to Dr. Klaus Schwarz for helpful advice and criticism in the course of these investigations; to C. E. Emery for the preparation of stachydrin hydrochloride; to Mary A. Gray for the blood studies; to Ligia Ortiz, Helen Hood, and Esther Hurley for preparation of the diets; and to Mary Stull and Woodrow Duvall for the care of the animals.

LITERATURE CITED

ARTOM, C., AND W. E. CORNATZER 1948 The action of ethanolamine, methylethanolamine and dimethylethanolamine on lipide metabolism. J. Biol. Chem., *176:* 949.

BERNHEIM, F., AND M. L. C. BERNHEIM 1933 Oxidation of acetylcholine by tissues. Am. J. Physiol., *104:* 438.

CASSELMAN, W. G. B., AND G. R. WILLIAMS 1954 Choline deficiency in the guinea pig. Nature, *173:* 210.

CORNATZER, W. E. 1954 Toxicity of choline and dimethylethanolamine in the guinea pig and the rat. Proc. Soc. Exp. Biol. Med., *85:* 642.

DUBNOFF, J. W. 1949a Utilization of choline and betaine methyl in the guinea pig. Arch. Biochem., *22:* 474.

———— 1949b The role of choline oxidase in labilizing choline methyl. Ibid., *24:* 251.

DU VIGNEAUD, V., S. SIMMONDS, J. P. CHANDLER, A. W. MOYER AND M. COHN 1946 The role of dimethyl- and monomethylaminoethanol in transmethylation reactions in vivo. J. Biol. Chem., *164:* 603.

DU VIGNEAUD, V., S. SIMMONDS, J. P. CHANDLER AND M. COHN 1946 A further investigation of the role of betaine in transmethylation reactions in vivo. Ibid., *165:* 639.

GRIFFITH, W. H. 1954 Effects of deficiency (choline). Chapter 5, p. 63. The Vitamins, Vol. II, Academic Press, Inc., New York.

HANDLER, P. 1949 Response of guinea pigs to diets deficient in choline. Proc. Soc. Exp. Biol. Med., *70:* 70.

HANDLER, P., AND F. BERNHEIM 1942 The choline oxidase activity of fatty livers. J. Biol. Chem., *144:* 401.

HOVE, E. L., AND D. H. COPELAND 1954 Progressive muscular dystrophy in rabbits as a result of chronic choline deficiency. J. Nutrition, *53:* 391.

HOVE, E., D. H. COPELAND AND W. D. SALMON 1954 Choline deficiency in the rabbit. Ibid., *53:* 377.

JUKES, T. H. 1947 Choline. Ann. Rev. Biochem., *16:* 193.

JUKES, T. H., AND J. J. OLESON 1945 Choline-like nutritional effect of dimethylaminoethanol. J. Biol. Chem., *157:* 419.

JUKES, T. H., J. J. OLESON AND A. C. DORNBUSH 1945 Observations on monomethylaminoethanol and dimethylaminoethanol in the diet of chicks. J. Nutrition, *30:* 219.

KENSLER, C. J., AND H. LANGEMANN 1954 Metabolism of choline and related compounds by hepatic tissue from several species including man. Proc. Soc. Exp. Biol. Med., *85:* 364.

MANTEL, N. 1951 Rapid estimation of standard error of means for small samples. Am. Statistician, *5:* 26. (Oct.)

MCKITTRICK, D. S. 1947 The interrelations of choline and methionine in growth and the action of betaine in replacing them. Arch. Biochem., *15:* 133.

———— 1948 The interrelations of choline and glycine betaine in the growth of the chick. Ibid., *18:* 437.

PILGERAM, L. O., E. M. GAL, E. N. SASSENRATH AND D. M. GREENBERG 1953 Metabolic studies with ethanolamine-1, 2-C^{14}. J. Biol. Chem., *204:* 367.

PILGERAM, L. O., AND D. M. GREENBERG 1954 Susceptibility to experimental atherosclerosis and the methylation of ethanolamine-1,2-C^{14} to phosphotidyl choline. Science, *120:* 760.

PLATT, A. P., AND R. R. PORTER 1947 Influence of choline and ethanolamine on the rate of formation of phospholipids in the liver. Nature, *160:* 905.

REID, M. E. 1954 Nutritional studies with the guinea pig. B-vitamins other than pantothenic acid. Proc. Soc. Exp. Biol. Med., *85:* 547.

REID, M. E., AND G. M. BRIGGS 1953 Development of a semi-synthetic diet for young guinea pigs. J. Nutrition, *51:* 341.

STETTEN, D., JR 1941 Biological relationships of choline, ethanolamine and related compounds. J. Biol. Chem., *140:* 143.

WELLS, I. C. 1954 Oxidation of choline-like substances by rat liver preparations. Inhibitors of choline oxidase. Ibid., *207:* 575.

THE THREONINE REQUIREMENT OF THE NORMAL INFANT [1,2]

EDWARD L. PRATT, SELMA E. SNYDERMAN,[3] MUNG W. CHEUNG,[4] PATRICIA NORTON AND L. EMMETT HOLT, JR.

Department of Pediatrics, New York University College of Medicine, New York City

AND

ARILD E. HANSEN AND THEODORE C. PANOS

Department of Pediatrics, University of Texas, Galveston

SIX FIGURES

(Received for publication November 29, 1954)

INTRODUCTION

The need of precise knowledge of nutritional requirements is an obvious one. Such information is essential for the recognition and intelligent repair of defective dietary situations. It is of particular importance in disturbances of the digestive tract where there are limitations in the amount of food that can be given, both enterally and parenterally. Information in regard to human requirements for amino acids has been slow in coming, for the production of an experimental diet deficient in a single amino acid which could be supplemented by known amounts of the amino acid in question involves particular difficulties. In approaching this problem several types of experimental diets have been employed:

[1] This work was supported in part by the U. S. Department of Agriculture under Contract A-1s-30942; the opinions expressed in this report are those of the authors and do not necessarily reflect those of the Department.

[2] A preliminary report of this work was published in Federation Proceedings *12:* 429, 1953 and in the Transactions of the American Pediatric Society, Am. J. Dis. Child., *86:* 324, 1953.

[3] H. R. Scheider Research Fellow, New York University.

[4] Playtex Park Research Fellow, New York University.

1 The use of natural proteins deficient in some particular amino acid. This method has very limited applicability, for nature has been singularly unobliging in providing a variety of such proteins.

2 The use of chemically degraded proteins or protein hydrolysates. A number of procedures are known which destroy one or more specific amino acids. Diets constructed from such degraded preparations have the advantage that the amino acids are for the most part present as natural isomers and that the unessential amino acids are present, avoiding the necessity of their synthesis. There is, however, the disadvantage that the nature and possible effects of the degraded fragments are unknown.

3 The use of synthetic amino acid mixtures. Such diets have the advantage that all the ingredients are accurately known, but the disadvantage that some of the amino acids have had to be supplied as DL forms. The extent to which the unnatural isomers were utilized and the unknown effects on non-utilizable amino acids introduced uncertainties in the experiment. Furthermore, the fact that the non-essential amino acids are for the most part omitted, requiring their synthesis from essential amino acids likewise detracts from the value of the data.

A 4th method of approaching amino acid requirements has been *to calculate the intake of amino acids on known diets compatible with health.* This method gives at best only an outside figure; one can say only that the requirement must be less than the intake so calculated.

These difficulties and sources of error can be avoided by the use of a diet in which the protein moiety is provided by a mixture of pure amino acids — both non-essentials and essentials — all in the form of the natural optical isomers. In recent years the L-forms of all the common dietary amino acids have become available and we have therefore employed such a diet in the present experiments which were designed to evaluate the threonine requirement of the normal human infant.

EXPERIMENTAL

Plan of study. This consisted in constructing a synthetic basal diet, the nitrogenous moiety of which was provided by a mixture of 18 L-amino acids. The composition of the amino acid mixture was based on that of breast milk, the figures used being for the most part those compiled by Macy et al. ('53) modified in a few instances by more recent data of Cheung et al. ('53). When it had been established that this diet permitted normal growth performance the threonine was removed from the mixture and then reintroduced in a stepwise fashion. It was replaced by an equivalent weight of glycine. By altering the threonine intake in this manner the minimum quantity compatible with normal growth performance was ascertained. The criteria of normality applied were the weight curve, nitrogen retention and plasma protein levels. Exploratory studies were also made of the excretion pattern of free amino acids in the urine in the hope that this might provide a useful criterion of adequacy of amino acid intake.

Preparation and administration of the experimental diet. The composition of the basal diet is given in table 1.

The diet was prepared as follows: the dry ingredients with the exception of the vitamins were weighed out and mixed. The fat was brought to the melting point and then mixed into the dry ingredients with an electric mixer. A known volume of warm water was added, the electric mixing being continued until an homogenous preparation was obtained. This gave a feeding of cereal-like consistency which was fed by spoon to the older infants studied. To the younger infants this was mixed with additional water and fed as a liquid formula. Special care was used to insure complete feeding by washing the bottle with warm water three times and feeding this to the baby. As a rule the intake was held to approximately 125 Cal. per kilogram. However, in some instances 150 Cal. per kilogram were given. All infants took the feeding readily and seemed to like it.

TABLE 1

Composition of experimental diet

	GRAMS	% OF TOTAL CALORIES
L-Amino acid mixture [1]	100	12
L-Alanine	2.67	
L-Arginine	4.58	
L-Aspartic acid	8.78	
L-Cystine	2.14	
L-Glutamic acid	17.56	
L-Glycine	2.06	
L-Histidine	1.76	
L-Isoleucine	6.11	
L-Leucine	11.76	
L-Lysine hydrochloride	7.10	
L-Methionine	1.68	
L-Phenylalanine	4.89	
L-Proline	6.11	
L-Serine	5.34	
L-Threonine [2]	4.58	
L-Tyrosine	4.58	
L-Tryptophan	1.68	
L-Valine	6.64	
Hydrogenated vegetable fat [3]	160	43
Dextrimaltose [4]	375	45
Mineral mixture [5]	22.3	
B Vitamin mixture [6]		10.0 ml per day
Vitamins A, C and D supplied as Trivisol [4]		0.6 ml per day

[1] The amino acids were obtained from various commercial sources. They were checked for purity by column chromatography and specific rotation. In some instances further purification was necessary.

[2] Supplied by courtesy of Winthrop-Stearns, Inc.

[3] Crisco, supplied by courtesy of Procter and Gamble.

[4] Supplied by courtesy of Mead Johnson and Co.

[5] The composition of the mineral mixture was as follows: NaCl 18.9, $CaHPO_4$ (anhydrous) 25.4, $MgSO_4$ (anhydrous) 6.8, $KHCO_3$ 44.4, KCl 2.88, Fe_3 Citrate 2.21, $CuSO_4$ (anhydrous) 0.24, $MnSO_4$ (anhydrous) 0.15, KI 0.015, NaF 0.03%.

[6] The composition of the B vitamin mixture was as follows: thiamine 0.38, riboflavin 2.0, nicotinamide 0.85, calcium pantothenate 3.5, pyridoxine 0.67, inositol 180, para-aminobenzoic acid 0.5, folic acid 0.05, choline chloride 147, biotin 0.03, vitamin B_{12} 0.015 mg.

The B vitamin mixture and one of vitamins A, C and D [5] were given once a day, being added to the 10 A.M. feeding after it had been brought to the proper temperature.

Chemical and metabolic data. These were obtained on 4 of the 8 subjects studied. The infants were placed on metabolism frames (Reynolds, '43) permitting separate collection of urine and feces and were supervised by experienced metabolism nurses. Metabolism periods were of 4 days duration in every instance and were preceded by three days upon the particular diet studied.

Stools for metabolic periods were marked with carmine. They were kept on ice, weighed and a wet aliquot taken for nitrogen analysis after thorough mixing.

Twenty-four-hour urine specimens were collected and separately preserved with 10% thymol-chloroform (3 ml per 100 ml of urine). They were kept on ice or in a frozen state until analyzed.

Blood for protein analysis was drawn at the end of each metabolic period, allowed to clot and the serum separated, stored in sealed tubes and analyzed within 24 hours. Amino nitrogen analyses were carried out at biweekly intervals on heparinized blood.

Analytical procedures. Urine. Total nitrogen was determined by the Kjeldahl procedure recommended by Hiller et al. ('48). Alpha amino nitrogen was determined by the gasometric ninhydrin-carbon dioxide method of Van Slyke et al. ('43). Amino acids were determined by ion exchange resin column chromatography using the procedure of Moore and Stein ('51). Creatinine was determined by the procedure of Bonsnes and Taussky ('45).

Stools. Nitrogen was determined on aliquots by the Kjeldahl procedure of Hiller et al. ('48).

Blood. Total serum protein was determined by the biuret test — a direct colorimetric measurement being made of the red-purple color formed by the treatment of proteins with a dilute alkaline copper solution. Weichselbaum's biuret re-

[5] Trivisol, supplied by courtesy of Mead Johnson and Co.

agent (Weichselbaum, '46) was employed. Albumin was separated from the globulins by the use of 23% sodium sulfite, the non-precipitated protein being determined by the biuret reaction and the concentration of globulin obtained by difference.

Subjects. Eight infants, believed to be metabolically normal were used in the present study. Some of these were hospitalized for social problems, others had been admitted to the hospital for some acute disease from which they had recovered at the time the studies were commenced. They were under 6 months of age when studied. Individual protocols were as follows:

Baby He., ♂, white. Born 10/10/52. Birth weight 3.47 kg. Well baby. Remained in John Sealy Hospital, Galveston because of a social problem. Started on study at the age of two weeks when he was 0.21 kg above his birth weight.

Baby Sa., ♂, white. Born 5/26/52. Birth weight 2.64 kg. Admitted to John Sealy Hospital, Galveston on 8/14/52 because of malnutrition and a social problem. After a period of observation a diagnosis of pyloric stenosis was made and operation was performed on 9/22/52. He made an uneventful recovery and started to gain weight well. He was 4½ months old and weighed 4.40 kg when the study was begun.

Baby St., ♂, white. Born 7/22/52. Birth weight 3.54 kg. Admitted to John Sealy Hospital, Galveston with a diagnosis of meningitis. He responded well to specific therapy and was afebrile after 36 hours. He was placed on study after he had been convalescent for two weeks and was making a satisfactory weight gain. At this time he was 3½ months old and weighed 6.60 kg.

Baby Ne., ♀, colored. Born 8/19/53. Birth weight 2.68 kg. Remained in Bellevue Hospital, New York City because mother had tuberculosis. Child not exposed and remained well. Was 111 days old and weighed 5.32 kg when started on full amino acid diet at 150 Cal. per kilogram. Weight gain for previous two weeks on milk diet was 0.49 kg.

Baby Ca., ♂, colored. Born 10/10/53. Birth weight 3.49 kg. Sixty days old, weight 4.11 kg when started on the experimental diet, which was fed at 150 Cal. per kilogram. Well baby, admitted to Bellevue Hospital because of social problem. Weight gain in two weeks prior to study 0.50 kg.

Baby Mo.–A (twin) ♀, colored. Born 10/4/53. Birth weight 1.84 kg. Remained in Bellevue Hospital because of a social problem. Was 68 days old, weight 3.09 kg when started on experimental diet which was fed at level of 150 Cal. per kilogram. Uneventful clinical course, discharged 11/13/53. Weight gain in two weeks preceding the study 0.45 kg.

Baby de la R., ♀, colored. Born 10/30/53, in Mt. Sinai Hospital, New York City. Birth weight 2.9 kg. Had more than regained birth weight when the study was commenced at age of 6 days. She then weighed 3.0 kg.

Baby Ro., ♂, white. Born 1/12/53. Birth weight 2.72 kg. Kept in John Sealy Hospital, Galveston for social reasons. Was 18 days old, weight 3.02 kg when started on experimental diet. Uneventful course. Was discharged before completion of study.

PRELIMINARY STUDIES

Before instituting studies of the threonine requirement it was thought advisable to test the adequacy of the basal diet, to determine in particular if young infants would thrive on a diet in which nitrogen was supplied altogether in the form of amino acids. Two premature infants in the Bellevue Hospital premature unit were selected for this study and both were fed from the first few days of life on the basal diet described above at a level of 125 Cal. per kilogram. Both of these infants thrived on the diet, exhibiting every sign of health. Their weight curves corresponded with the expected curve for their birth weight as defined by Dancis et al. ('48). The weight curve of one of these infants is shown in figure 1. It may be noted that in these preliminary experiments as well as in those reported below a normal weight curve often re-

quired a caloric intake of 25% above the usual figures, indicating that even a complete amino acid mixture is less efficient than whole protein, and observation that has frequently been made in animals, and in humans by Rose ('49).

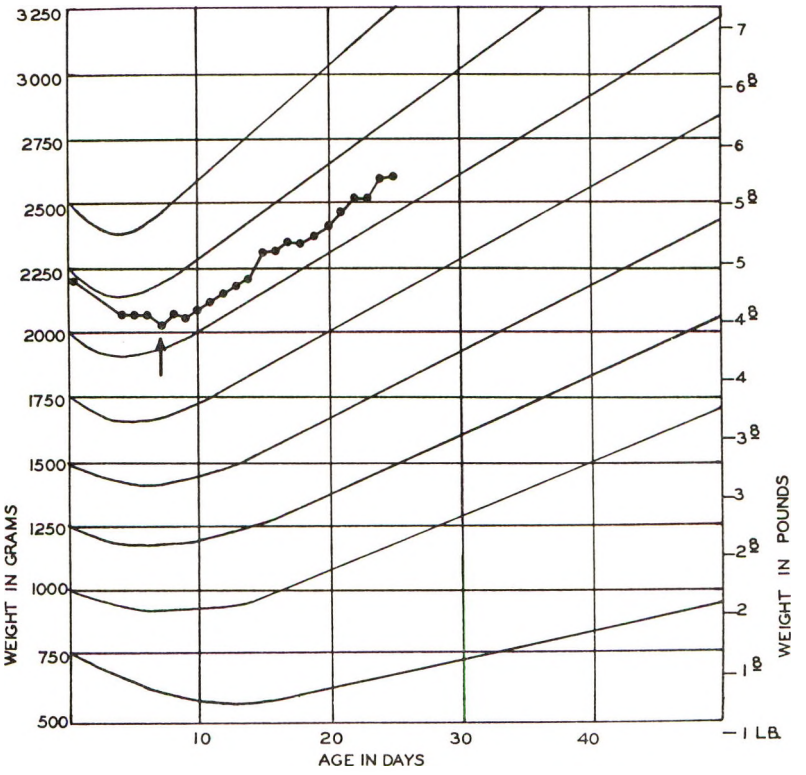


Fig. 1 Growth curve of infant on basal diet. Special formula: amino acids, 8%; fat, 47%; carbohydrate, 42% furnished 3.0 gm of amino acids per kilogram. Arrow indicates start of amino acid formula. Baby Re.

OBSERVATIONS ON THE THREONINE REQUIREMENT

Having established the validity of our basal diet we next proceeded to study the effect of threonine withdrawal and supplementation. The results obtained in the several infants are shown graphically in figures 2 to 6.

Baby He. (fig. 2) failed to gain weight when threonine was removed from his diet. He gained subnormally when

given 30 mg of threonine per kilogram per day, but gained normally on 45 mg. Nitrogen retention decreased with the threonine intake, but there appeared to be a break in the nitrogen curve when plotted against threonine intake (fig. 3)

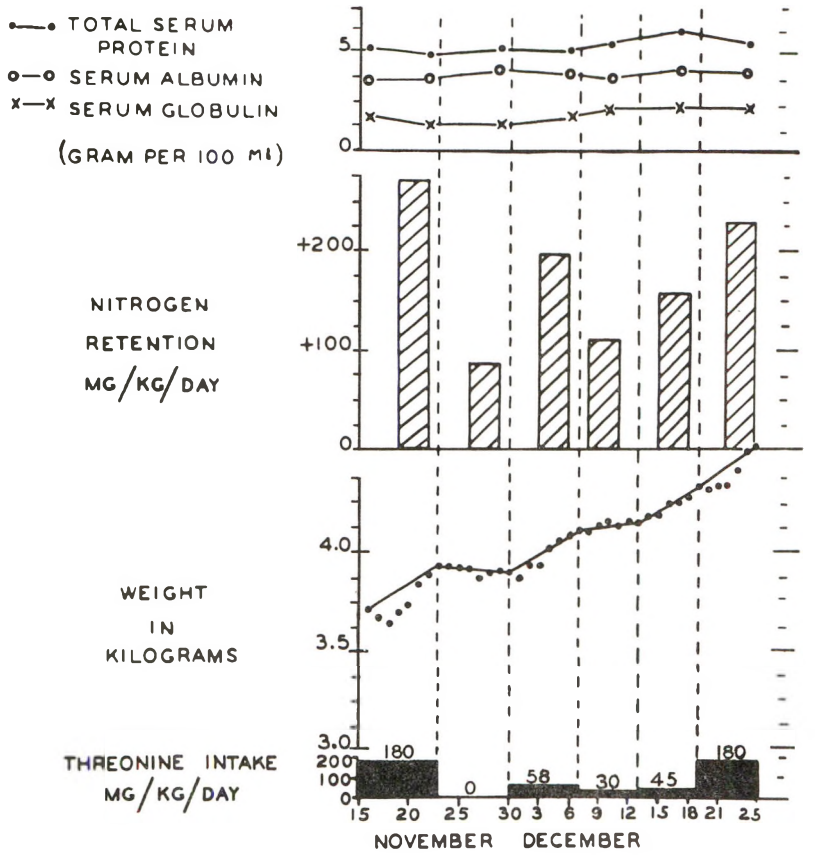


Fig. 2 Threonine requirement of the normal infant, Baby He, two weeks old.

when the latter fell below 58 mg per kilogram per day. There was no definite change in the plasma proteins with low threonine intakes. It seems reasonable to conclude that the minimum threonine requirement for this baby was between 45 and 58 mg per kilogram per day.

Baby Sa., when given no threonine, ceased to gain weight and to retain nitrogen (fig. 4). His weight gain appeared to be slightly impaired on 48 mg per kilogram per day, but normal on 70 mg. The break in nitrogen retention occurred at a point below 48 mg per kilogram per day. Plasma globulin showed a tendency to reduction in the low threonine intake periods, but no well-defined break. One may conclude from

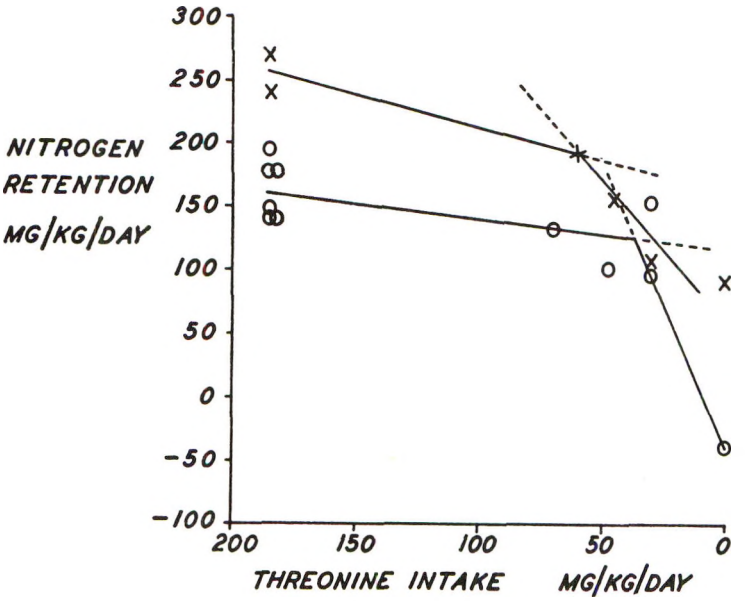


Fig. 3 Relation between threonine intake and nitrogen retention, Baby Sa = 0; Baby He = x.

these data that the threonine requirement of this infant lay between 48 and 70 mg per kilogram per day, probably closer to the first figure.

Baby St. (fig. 5), when reduced to a threonine intake of 47 mg per kilogram per day, continued to gain weight at practically the normal rate. His plasma globulin did not fall and his nitrogen retention, though less than on the complete basal diet showed no sharp decrease. It appears that 47 mg per kilogram per day was barely adequate for this subject.

Baby Ne. gained weight normally on a complete amino acid intake providing 176 mg of threonine per kilogram per day. A reduced intake providing 44 and 59 mg failed to permit this, although an intake of 87 mg per kilogram per day restored normal growth. The requirement for this baby apparently lay between 59 and 87 mg per kilogram per day.

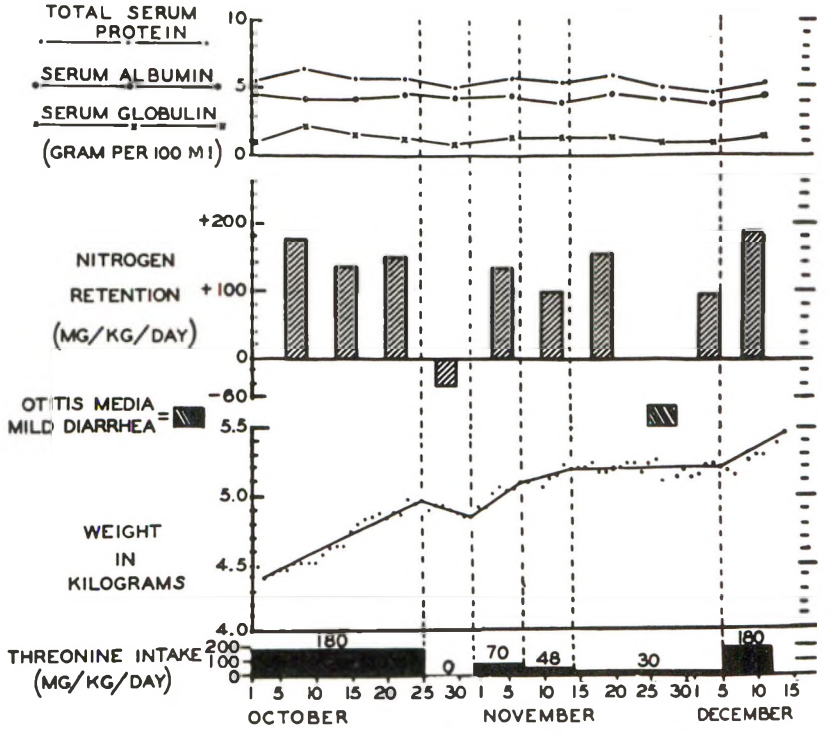


Fig. 4 Threonine requirement of the normal infant, Baby Sa, 4½ months old.

Baby Mo.–A In this infant a reduction in threonine intake from 176 to 45 mg per kilogram per day reduced the gain in weight a minimum amount. A normal weight curve was obtained with 60 mg. The requirement hence appeared to be between 45 and 60 mg per kilogram per day.

Baby Ca. gained normally on a milk diet providing 125 Cal. per kilogram. When placed on the basal amino acid diet, 150 Cal. per kilogram were needed to permit normal growth.

On this caloric intake a reduction of threonine to 44.5 mg per kilogram per day caused impaired weight gain. The requirement of this baby therefore appeared to be greater than this figure.

Baby de la R. This baby gained normally on a threonine intake of 175 mg per kilogram per day, but failed to gain weight on 45 mg.

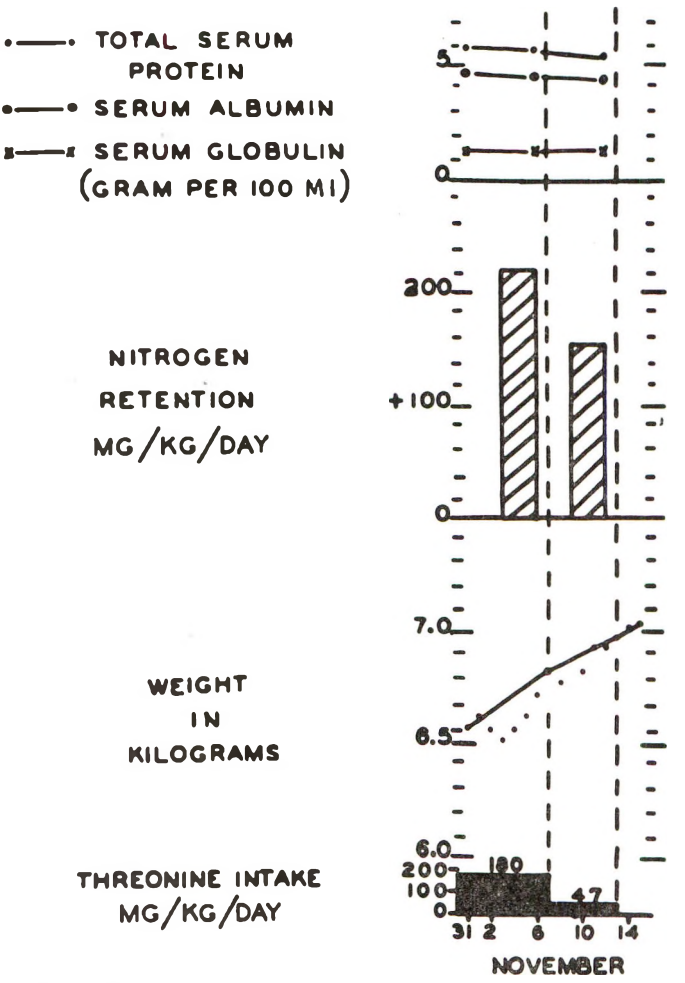


Fig. 5 Threonine requirement of the normal infant, Baby St, 3½ months old.

Baby Ro. (fig. 6) when given no threonine lost weight, failed to retain an adequate quantity of nitrogen and excreted an excess of nitrogen in the urine. It was not possible to complete the study on this baby inasmuch as he was discharged unexpectedly for social reasons. All that can be said was that he showed evidence of a threonine requirement

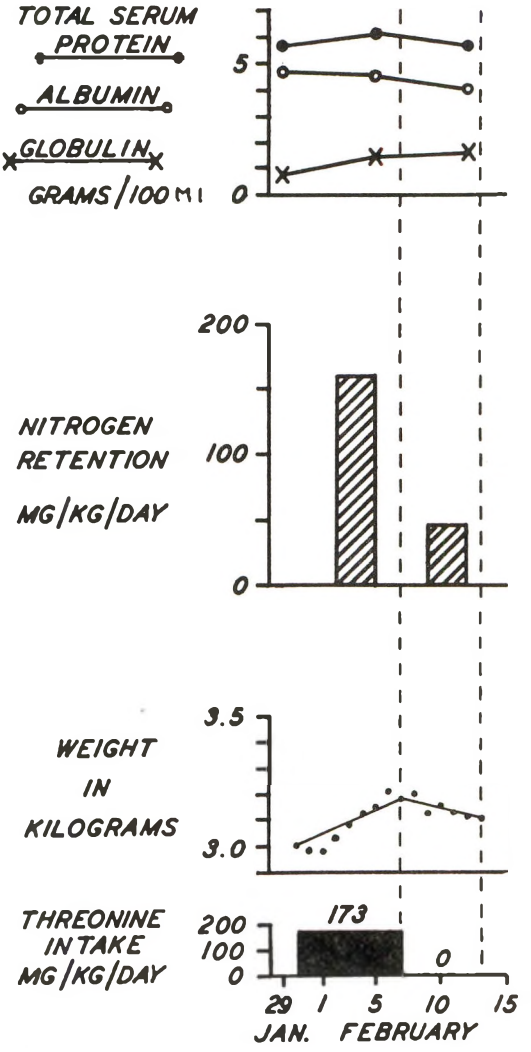


Fig. 6 Threonine requirement of the normal infant, Baby Ro, 18 days old.

greater than zero. A summary of the threonine intake in these studies is given in table 2.

It thus appears that although two subjects maintained themselves on 45 and 47 mg respectively, it was necessary to reduce the threonine intake below 60 mg per kilogram per day to obtain evidence of threonine deficiency for most babies. In one subject (Ne.) the minimum requirement may have been above 60 mg. However, 60 mg per kilogram per day may be regarded as the usual threonine requirement of infants under 6 months of age.

TABLE 2

Threonine intake of each infant

SUBJECT	THREONINE INTAKE	
	Inadequate	Adequate
	mg/kg/day	*mg/kg/day*
He	30	45
Sa	48	70
St		47
Ne	59	87
Mo – A	45	60
Ca	44.5	
de la R	45	
Ro	0	

CLINICAL OBSERVATIONS ON INFANTS FED THREONINE-DEFICIENT DIETS

The arrest of weight gain associated with threonine-deficient diets has already been referred to. Three of our subjects (He., Sa. and Ro.) were maintained for a period of one week on a diet containing no threonine at all. In all three of these subjects there developed glossitis and a reddening of the buccal mucosa, most marked near the junction with the vermilion border of the lips. These symptoms subsided promptly when threonine was added to the diet. Whether this finding was coincidental or a direct consequence of threonine deficiency cannot be stated with certainty at the present time.

METABOLIC OBSERVATIONS

The general metabolic data obtained on the 4 subjects studied are given in tables 3, 4, and 5. It is clear that threonine deficiency leads to increased loss of nitrogen in the urine which may be sufficient to bring about a negative nitrogen balance. In this increased azoturia amino acid nitrogen plays little part; indeed in subjects He. and St. it showed no rise whatever. The effect of threonine withdrawal on the nitrogen balance was more striking in the case of the older subject Sa., in whom alone it induced a negative nitrogen balance. The younger and more rapidly growing infants continued to retain some nitrogen in the face of this dietary deficiency.

The deficient diet failed to influence the absorption of nitrogen in any detectable way. The serum proteins were noticeably affected only in the case of Sa., in whom a reduction of globulin appeared to result from threonine deficiency. There was no significant alteration in the amino nitrogen of the blood.

Of particular interest were observations on the amino acid excretion in the urine. Ion exchange resin column chromatograms were run on subjects He., Sa. and Ro. in a control period with full threonine intake and in a period with no threonine. The findings are shown in table 6. The free amino acids identified by this method constituted about two thirds of the total amino nitrogen as determined by the Van Slyke procedure. Despite the increased nitrogen loss in the deficiency periods, no consistent aminoaciduria occurred. An examination of the amino acid excretion pattern reveals considerable individual variation; nevertheless certain changes stand out. There is a consistent reduction in threonine output in the threonine-deficient periods. There is also an increased output of glycine in the threonine-deficient periods which is not surprising in view of the fact that threonine was replaced by glycine. Other changes of possible significance are an increased output of histidine and a decreased output of leucine. These observations suggest that threonine deficiency is reflected in the threonine excretion in the urine. The reduction in

TABLE 3

Baby He — Metabolic observations

		PERIOD					
		I	II	III	IV	V	VI
DATE (INCLUSIVE)		11/17–11/20/52	11/24–27	12/1–4	12/8–11	12/15–18	12/22–25
Average weight, kg		3.75	3.87	4.0	4.1	4.25	4.4
Threonine intake, mg/kg/day		*180*	*0*	*58*	*30*	*45*	*180*
Nitrogen intake, gm/4 days		7.32	7.18	7.52	7.88	7.88	8.24
Nitrogen in urine, gm/4 days		2.19	5.24	3.43	5.18	4.26	3.19
Nitrogen in stool, gm/4 days		1.07	0.56	0.96	0.90	0.97	0.82
Nitrogen retention, mg/kg/day		270	89	196	111	156	240
Urine creatinine, mg/day		42.3	41.5				
Urine amino N, mg/day		22.6	20.5	24.9	22.7	25.1	29.2
Serum proteins, gm/100 ml							
	PREPERIOD						
• *Total*	*5.1*	4.8	5.4	4.9	5.5	5.9	5.2
Albumin	*3.5*	3.6	4.1	3.3	3.6	3.9	3.7
Globulin	*1.6*	1.2	1.3	1.6	1.9	2.0	1.5
Plasma amino N, mg/100 ml				4.30		3.01	4.47

TABLE 4

Baby Sa — Metabolic observations

		PERIOD											
		I	II	III	IV	V	VI	VII	VIII [1]	IX	X	XI [2]	XII
DATE (INCLUSIVE)	1952	10/6–9	10/13–16	10/20–23	10/27–30	11/3–6	11/10–13	11/17–20	11/24–27	12/2–5	12/8–11	12/15–18	12/22–25
Average weight, kg		4.53	4.69	4.88	4.90	5.0	5.1	5.2	5.2	5.2	5.3	5.6	5.7
Threonine intake, mg/kg/day		*180*	*180*	*180*	*0*	*70*	*48*	*30*	*30*	*30*	*180*	*180*	*180*
Nitrogen intake, gm/4 days		8.44	9.00	9.20	9.44	9.56	9.60	9.93		9.92	10.12	10.69	10.90
Nitrogen in urine, gm/4 days		4.76	5.43	5.49	8.33	6.01	6.94	5.89		7.27	5.44	6.56	5.58
Nitrogen in stool, gm/4 days		0.44	1.02	0.80	1.19	0.90	0.60	0.79		0.69	0.88	1.04	0.92
Nitrogen retention, mg/kg/day		179	139	149	— 41	133	101	156		94	179	138	193
Urine creatinine, mg/day					68			66					
Urine amino N, mg/day		22.3	23.3	32.2	19.8	23.6	21.3	20.7	29.3	23.5	20.0	27.4	26.9
Serum proteins, gm/100 ml													
	PREPERIOD												
Total	5.5	6.4	5.8	5.8	5.0	5.7	5.2	5.8	4.9	4.5	5.5	5.5	5.8
Albumin	4.4	4.2	4.2	4.5	4.2	4.4	3.2	4.6	4.1	3.7	4.2	4.2	4.3
Globulin	1.1	2.2	1.6	1.3	0.8	1.3	1.3	1.2	0.8	0.8	1.3	1.3	1.3
Plasma amino N, mg/100 ml			4.23		5.64	4.16	5.72	3.58		3.14	3.53		

[1] Otitis media and mild diarrhea during this period. Diet continued, but taken off metabolism.

[2] Otitis media during this period.

TABLE 5

Metabolic observations

	BABY ST			BABY RO		
	PREPERIOD	PERIOD		PREPERIOD	PERIOD	
		I	II		I	II
DATE (INCLUSIVE)		1952			1953	
		11/3–6	11/10–13		2/2–5	2/9–12
Average weight, kg		6.6	6.9		3.1	3.1
Threonine intake, mg/kg/day		*180*	*47*		*173*	*0*
Nitrogen intake, gm/4 days		12.56	12.76		5.80	6.00
Nitrogen in urine, gm/4 days		5.83	7.46		3.16	4.95
Nitrogen in stool, gm/4 days		0.93	0.86		0.66	0.48
Nitrogen retention, mg/kg/day		222	152		160	46
Urine creatinine, mg/day	63.6		70.2	40.3	37.0	
Urine amino N, mg/day	28.3	36.9	42.7	26.2	18.5	24.6
Serum proteins, gm/100 ml						
Total	6.0	5.7	5.4	5.6	6.1	5.7
Albumin	4.8	4.6	4.2	4.7	4.5	4.0
Globulin	1.2	1.1	1.2	0.9	1.6	1.7
Plasma amino N, mg/100 ml	4.38	4.32	3.96	3.97	4.17	4.62

TABLE 6

Daily amino acid excretion in urine on threonine-deficient diet

	BABY He				BABY Sa				BABY Ro			
	PERIOD				PERIOD				PERIOD			
	I		II		III		IV		I		II	
THREONINE INTAKE, *mg/kg/day*	*180*		*0*		*180*		*0*		*180*		*0*	
AVERAGE WEIGHT, *kg*	3.7		3.87		4.88		4.90		3.1		3.1	
URINE AMINO ACIDS, *per day*	*mg*	*μmole*	*mg*	*μmole*	*mg*	*μmole*	*mg*	*μmole*	*mg*	*μmole*	*mg*	*μmole*
Alanine	5.3	59.5	5.3	59.5	6.5	73	7.3	82.0	7.2	82.0	7.9	80.8
Arginine	1.6	9.2	1.1	6.3	0	0	0	0	3.5	20.1	2.1	12.0
Aspartic acid	4.4	33.1	5.4	40.6	4.4	33.1	4.3	32.3	2.4	18.1	3.9	29.3
Cystine	0.8	3.3	1.6	6.7	0	0	0	0	2.1	8.7	3.3	13.7
Glutamic acid	8.3	56.5	1.8	12.2	12.1	82.3	5.5	37.4	5.0	34.0	3.4	23.1
Glycine	25.2	336	30.8	411	15.2	202.5	17.3	230.5	28.9	385.5	51.4	685.0
Histidine	7.0	45.1	11.2	72.1	13.2	85.0	18.1	116.5	10.8	69.6	14.5	93.4
Isoleucine	2.4	18.3	1.8	13.7	3.5	26.7	4.0	30.5	2.4	18.3	1.9	14.5
Leucine	2.3	17.5	0.6	4.6	3.1	23.6	2.3	17.5	2.3	17.5	1.5	11.4
Lysine complex [1]	17.0	116.1	16.7	114.0	21.2	145.0	20.7	141.5	17.8	121.7	20.2	138.0
Methionine	3.3	22.1	2.2	14.7	3.3	22.1	5.0	33.5	0	0	2.2	14.7
Phenylalanine	9.0	54.5	7.8	47.2	10.3	62.4	9.8	59.3	1.2	7.3	1.9	11.5
Proline	7.1	61.7	6.0	52.1	3.3	28.7	5.9	51.3	9.5	82.6	9.1	79.1
Serine (includes asparagine and glutamine)	20.9	199.0	16.6	158.0	20.1	191.0	20.1	191.0	6.5	61.8	15.4	146.5
THREONINE	*6.7*	*56.3*	*1.3*	*10.9*	*6.8*	*57.1*	*4.2*	*35.3*	*8.4*	*70.6*	*1.1*	*9.2*
Tryptophan	1.8	8.8	1.4	6.9	0	0	0	0	0	0	0	0
Tyrosine	2.4	13.2	2.5	13.8	2.7	14.9	2.2	12.1	3.4	18.8	3.2	17.7
Valine	1.8	15.4	2.0	17.1	2.8	23.9	2.6	22.2	1.7	14.5	1.6	13.6
Total identified amino acids	127.3	1125.6	116.1	1061.4	128.5	1071.3	129.3	1092.9	112.9	1029.9	144.4	1401.4
Urine volue, ml/day	273		319		655		638		503		511	
Urine total N, *gm/day*	0.547		1.31		1.27		1.96		0.79		1.24	
Free (identified on column) amino N, mg/day	15.8		14.9		15.0		15.3		14.4		19.6	
(% total N)	2.9		1.1		1.2		0.8		1.8		1.6	
Free amino N (Van Slyke), mg/day	22.65		20.5		32.2		22.9		18.5		24.6	
(% total N)	4.2		1.6		2.5		1.2		2.3		2.0	

[1] This may include small and variable amounts of other amino acids — e.g. 1-methyl histidine and ornithine.

threonine output on a threonine-deficient diet was, however, not observed by Leverton and her coworkers ('52) in studies on adults.

In comparing the amino acid excretion pattern which we observed in the control periods with data in the literature (Cooper et al., '50; Eckhardt and Davidson, '49; Dunn et al., '47) it is apparent that there is little agreement between workers who have employed different analytical methods. Our figures are, however, in fairly close agreement with those of Stein ('53) who studied the amino acid excretion of adults by column chromatography, with two exceptions: they reported considerably more histidine and considerably less lysine in the urine. Whether this difference is due to the age or diet of the subjects remains to be ascertained.

SUMMARY

In studies designed to evaluate the threonine requirement of normal infants a synthetic diet was employed which provided nitrogen in the form of a mixture of 18 essential and non-essential amino acids, all as the natural L-isomers. It was found that infants would gain weight normally and exhibit every sign of health on such a diet, although a somewhat higher than average caloric intake was at times required.

By graded reduction of the threonine intake, threonine being replaced by glycine, the minimum threonine requirement was determined. This was found to be approximately 60 mg per kilogram per day for infants between one and 6 months of age.

A deficiency of threonine was associated with failure to gain weight and with impaired retention of nitrogen. There was increased loss of nitrogen in the urine, but no increased amino aciduria. Certain changes in the amino acid excretion pattern were observed, notably a decreased threonine excretion.

ACKNOWLEDGEMENTS

We wish to express our indebtedness to Eileen Hasselmyer, R.N. for her careful nursing supervision of these patients

and to Miriam Katz, R.N. who assisted her; to A. L. McDowell who performed the nitrogen determinations and to Dr. Hilda Wiese for technical advice.

LITERATURE CITED

BONSNES, R. W., AND H. H. TAUSSKY 1945 On the colorimetric determination of creatinine by the Jaffe reaction. J. Biol. Chem., *158:* 581.

CHEUNG, M. W., E. L. PRATT AND D. I. FOWLER 1953 Total amino acid composition in mature human milk. Pediatrics, *12:* 353.

COOPER, A. M., R. D. ECKHARDT, W. W. FALOON AND C. S. DAVIDSON 1950 Investigation of aminoaciduria in Wilson's disease: Demonstration of a defect in renal function. J. Clin. Invest., *29:* 265.

DANCIS, J., J. R. O'CONNELL AND L. E. HOLT, JR. 1948 A grid for recording the weight of premature infants. J. Pediat., *33:* 570.

DUNN, M. S., M. N. CAMIEN, S. SHANKMAN AND H. H. BLOCK 1947 Urinary excretion of 12 amino acids by normal male and female subjects measured microbiologically. Arch. Biochen., *13:* 207.

ECKHARDT, R. D., AND C. S. DAVIDSON 1949 Urinary excretion of amino acids by a normal adult receiving diets of varied protein content. J. Biol. Chem., *177:* 687.

HILLER, A., J. PLAZIN AND D. D. VAN SLYKE 1948 A study of conditions for Kjeldahl determination of nitrogen in proteins. J. Biol. Chem., *176:* 1401.

LEVERTON, R. M., M. R. GRAM, E. BRODOVSKY, A. MITCHELL AND M. CHALOUPKA 1952 Amino acid requirement of young women. I. Threonine. Fed. Proc., *11:* 448.

MACY, I. G., H. KELLY AND R. SLOAN 1953 The composition of milks. Bulletin of the National Research Council, No. 254.

MOORE, S., AND W. H. STEIN 1951 Chromatography of amino acids on sulfonatid polystyrene resins. J. Biol. Chem., *192:* 663.

REYNOLDS, T. 1943 Metabolism bed. Am. J. Nursing, *43:* 183.

ROSE, W. C. 1949 Amino acid requirement of man. Fed. Proc., *8:* 546.

STEIN, W. H. 1953 A chromatographic investigation of the amino acid constituents of normal urine. J. Biol. Chem., *201:* 45.

VAN SLYKE, D. D., D. A. MAC FAYDEN AND P. B. HAMILTON 1943 The gasometric determination of amino acids in urine by the ninhydrin carbondioxide method. J. Biol. Chem., *150:* 251.

WEICHSELBAUM, T. E. 1946 An accurate and rapid method for determination of proteins in small amounts of blood serum and plasma. Am. J. Clin. Path., Tech. Sect., *10:* 40.

THE PHENYLALANINE REQUIREMENT OF THE NORMAL INFANT [1,2]

SELMA E. SNYDERMAN,[3] EDWARD L. PRATT, MUNG W. CHEUNG,[4] PATRICIA NORTON AND L. EMMETT HOLT, JR.

Department of Pediatrics, New York University College of Medicine, New York City

AND

ARILD E. HANSEN AND THEODORE C. PANOS

Department of Pediatrics, University of Texas, Galveston

THREE FIGURES

(Received for publication November 29, 1954)

In an accompanying publication (Pratt et al., '55) we have discussed the difficulties in evaluating essential amino acid requirements in man and have pointed out that this can best be done by an experimental diet in which the protein moiety is provided by pure amino acids, the non-essential as well as the essential, all supplied as the natural L-isomers. The present report deals with studies in which this technique was employed to determine the phenylalanine requirement of the normal infant.

EXPERIMENTAL

The plan of study, general procedures, basal diet and analytical techniques employed were identical with those described in the accompanying study of the threonine require-

[1] This work was supported in part by the U. S. Department of Agriculture under Contract A-ls-30942. The opinions expressed are those of the authors and do not necessarily reflect the views of the Department.

[2] A preliminary report of this work was published in the Transactions of the American Pediatric Society, Am. J. Dis. Child., *86:* 324, 1953.

[3] H. R. Scheider Research Fellow, New York University.

[4] Playtex Park Research Fellow, New York University.

ment of the normal infant. The subjects consisted of 6 infants between one and 9 months of age. In two of these complete metabolism studies were made; in the remaining 4 only clinical observations, chiefly the weight curve, were used to evaluate the adequacy of the diet. Individual protocols of these subjects were as follows:

Baby He. ♂, white. Born 10/10/52. Birth weight 3.473 kg. Well baby. Remained in John Sealy Hospital, Galveston, because of a social problem. Prior to the present study had been used as a subject for study of threonine requirement. During the present study he was 2½ to 3½ months of age.

Baby Sa. ♂, white. Born 5/26/52. Birth weight 2.637 kg. Successfully operated on for pyloric stenosis, following which he was a subject for study of threonine requirement. The present study was commenced at the age of 6 months and was continued to the age of 9 months. During this period he suffered from two attacks of otitis media. The dietary period was continued until complete recovery from this complication occurred.

Baby Mo.–B, ♀, colored. One of twins, born 10/4/53. Birth weight 1.843 kg. Sixty-eight days old, weight 2.948 kg when started on experimental diet, fed 150 Cal. per kilogram. Uneventful clinical course, discharged from Bellevue Hospital (N. Y.) on 11/13/53. Hospitalized because of social problem. Weight gain in two weeks preceding study period 0.44 kg.

Baby Du. ♀, colored. Born 11/16/53. Birth weight 2.580 kg. Hospitalized in Bellevue Hospital because mother suffered from pulmonary tuberculosis. Child not exposed to mother and remained well. Thirty-one days old (weight 3.147 kg) when started on study. Weight gain for previous two weeks 0.310 kg.

Baby Th. ♂, colored. Born 11/30/53. Birth weight 2.424 kg. Remained in Bellevue Hospital because of social problem. Twelve days old (weight 2.792 kg) when started on control period of experimental diet. At this point he was 0.370 kg above his birth weight.

Baby Ri. ♀, colored. Born 11/9/53. Birth weight 2.438 kg. Remained in Bellevue Hospital because of a social problem. Developed loose stools on 12/2/53 which had ceased by 12/12/53. Started on study at age of 47 days at which

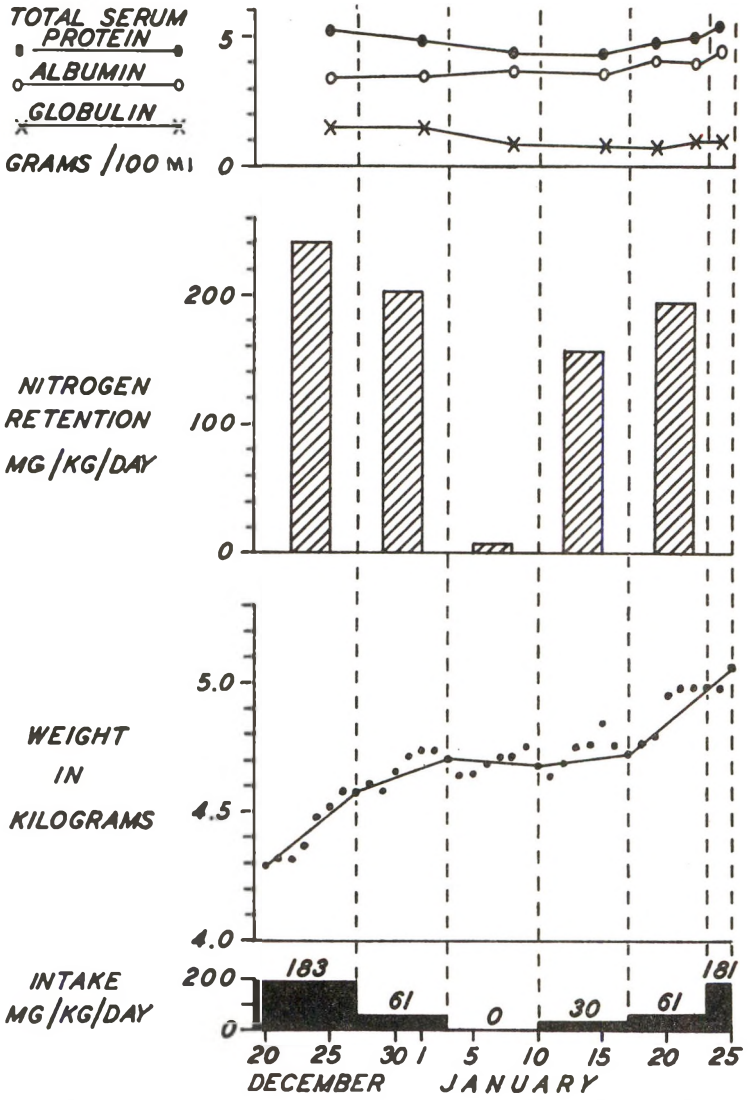


Fig. 1 Phenylalanine requirement of normal infant, Baby He two and one-half months old.

time she weighed 2.778 kg. She had gained 0.103 kg in the three days preceding the period of study.

OBSERVATIONS ON PHENYLALANINE REQUIREMENT

Baby He. (fig. 1) failed to gain weight and to retain nitrogen when phenylalanine was completely withdrawn; his serum globulin likewise fell. His weight gain on 30 mg of phenylalanine per kilogram per day was subnormal, and on 61 mg appeared to be borderline, adequate on one occasion and not on another. A sharp break in the nitrogen retention

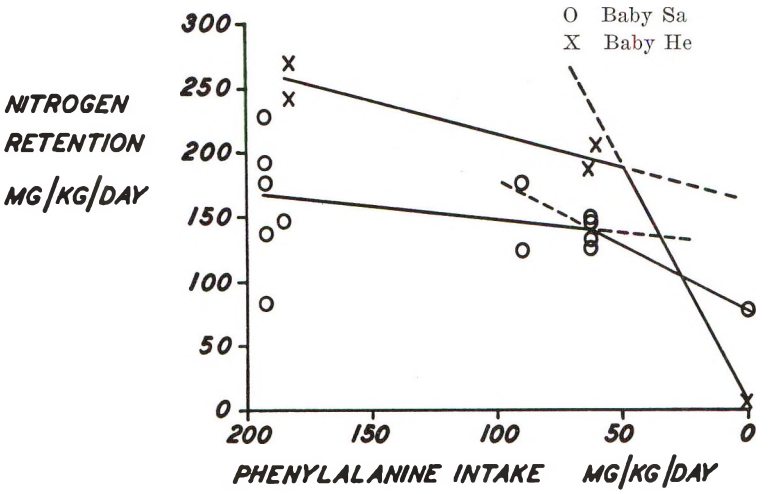


Fig. 2 Relation between phenylalanine intake and nitrogen retention.

occurred only below 61 mg (fig. 2). It would seem that 61 mg was barely inadequate for this baby.

Baby Sa. (fig. 3) showed an equivocal weight gain on 63 and 61 mg of phenylalanine per kilogram per day, adequate on one occasion and not on another, although a sharp drop in nitrogen retention occurred only below this level (fig. 2). With 91 mg the weight gain was adequate.

Baby Mo.–B showed inferior weight gain when his phenylalanine intake was reduced to 63 mg per kilogram per day, but gained normally on 94 mg.

Baby Du. was able to continue normal weight gain on 64 mg per kilogram per day.

Baby Th. was able to gain weight on as little as 47 mg of phenylalanine per kilogram per day.

Baby Ri., when given a diet containing 63 mg of phenylalanine per kilogram per day, continued to gain weight normally.

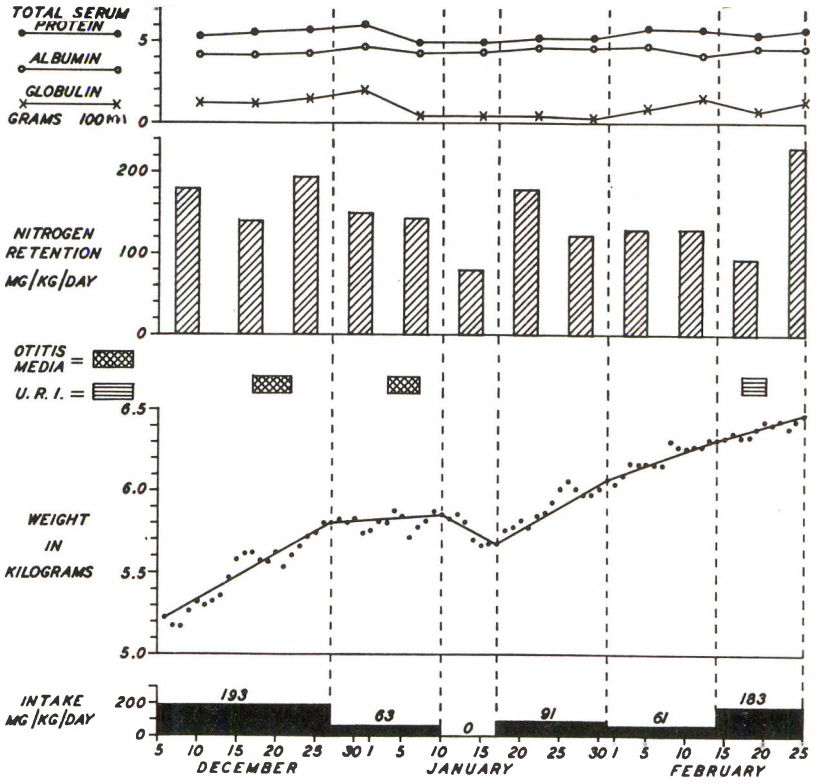


Fig. 3 Phenylalanine requirement of normal infant, Baby Sa, 6 months old.

A summary of these requirement studies is given in table 1.

It thus appears that although some babies may get along with as little as 47 mg of phenylalanine per kilogram per day, 61 to 63 mg appears to be marginal for others and 91 to 94 mg adequate.

CLINICAL OBSERVATIONS ON PHENYLALANINE DEFICIENT DIETS

In the two infants who were given diets completely free from phenylalanine, no clinical symptoms attributable to such deficiency were observed.

METABOLIC OBSERVATIONS

The general metabolic data obtained in subjects He. and Sa. are given in tables 2 and 3 and the data on specific amino acid excretion in table 4.

They demonstrate that phenylalanine deficiency, like that of threonine, impairs nitrogen balance by causing increased

TABLE 1

Phenylalanine intake of each infant

SUBJECT	PHENYLALANINE INTAKE	
	Inadequate	Adequate
	mg/kg/day	*mg/kg/day*
He.	61	
Sa.	63	91
Mo.-B	63	94
Du.		64
Th.		47
Ri.		63

nitrogen loss in the urine. Aminoaciduria is, however, more conspicuous than was the case with threonine deficiency. The deficiency of phenylalanine appeared to exercise a more unfavorable effect on the nitrogen balance in the younger of the two babies, the reverse of what was observed with threonine deficiency. The serum proteins, notably the globulins, were decreased in both subjects. There was no consistent change in the amino nitrogen of the blood.

The excretion pattern of the free amino acids in the urine showed some degree of variation which could not be readily attributed to the diet. In both subjects there was a striking drop in the phenylalanine excretion when this amino acid was withheld from the diet. One subject showed a decrease from

TABLE 2

Baby He. — Metabolic observations

	PERIOD						
	I	VI	VII	VIII	IX	X	XI [1]
DATE (INCLUSIVE)	1952 11/17–20	12/22–25	1953 12/29–1/1	1/5–8	1/12–15	1/19–22	1/23–25
Average weight, kg	3.75	4.40	4.65	4.69	4.77	4.90	5.05
Phenylalanine intake, mg/kg/day	*183*	*183*	*61*	*0*	*30*	*61*	*181*
Nitrogen intake, gm/4 days	7.32	8.24	8.63	8.81	9.00	9.00	4.60 [1]
Nitrogen in urine, gm/4 days	2.19	3.19	4.21	7.29	4.63	4.26	
Nitrogen in stool, gm/4 days	1.07	0.82	0.61	1.42	1.39	1.04	
Nitrogen retention, mg/kg/day	270	240	205	5.9	156	189	
Urine creatinine, mg/day	42.3			61.0			59.5
Urine amino N, mg/day	22.6	29.2	32.8	40.6	36.6	31.3	32.8
Serum proteins, gm/100 ml							
Total	4.8	5.2	4.9	4.5	4.3	5.1	5.3
Albumin	3.6	3.7	3.5	3.7	3.6	4.2	4.7
Globulin	1.2	1.5	1.4	0.8	0.9	0.9	0.8
Plasma amino N, mg/100 ml		4.47	3.81	4.67		4.63	

[1] Period of two days.

TABLE 3

Baby Sa. — Metabolic observations

	PERIOD												
	III	X	XI [2]	XII	XIII	XIV [2]	XV	XVI	XVII	XVIII	XIX	XX	XXI [1]
DATE (INCLUSIVE)	1952 10/20–23	12/8–11	12/15–18	12/22–25	1953 12/29–1–1	1/5–8	1/12–15	1/19–22	1/26–29	2/2–5	2/9–12	2/16–19	2/23–25
Average weight, kg	4.88	5.3	5.6	5.7	5.8	5.75	5.73	5.82	6.02	6.16	6.28	6.35	6.41
Phenylalanine intake, mg/kg/day	*185.5*	*193*	*193*	*193*	*63*	*63*	*0*	*91*	*91*	*61*	*61*	*183*	*183*
Nitrogen intake, gm/4 days	9.20	10.12	10.69	10.90	11.04	11.24	11.24	11.24	11.24	11.44	11.64	11.84	9.15 [1]
Nitrogen in urine, gm/4 days	5.49	5.44	6.56	5.58	6.69	6.23	8.39	6.49	7.49	7.31	7.60	8.63	5.06 [1]
Nitrogen in stool, gm/4 days	0.80	0.88	1.04	0.92	0.72	1.68	0.90	0.59	0.77	0.81	0.83	0.87	0.68 [1]
Nitrogen retention, mg/kg/day	149	179	138	193	150	145	80.5	179	124	134	128	92	230
Urine creatinine, mg/day							70.3			86.0		86.0	
Urine amino N, mg/day	32.2	20.0	27.4	26.9	25.2	29.7	27.5	21.8	26.9	29.6	28.9	33.4	22.9
Serum proteins, gm/100 ml													
Total	5.8	5.5	5.5	5.8	6.2	5.0	5.2	5.3	5.2	6.0	5.9	5.4	5.8
Albumin	4.5	4.2	4.2	4.3	4.2	4.5	4.6	4.5	4.7	4.9	4.3	4.6	4.5
Globulin	1.3	1.3	1.3	1.5	2.0	0.5	0.6	0.8	0.5	1.1	1.6	0.8	1.3
Plasma amino N, mg/100 ml		3.53			4.27		4.20			5.29		3.91	5.49

[1] Period of three days.

[2] Mild otitis media observed during this period.

TABLE 4

Amino acid excretion in urine on phenylalanine-deficient diet

	BABY HE.				BABY SA.			
	PERIOD				PERIOD			
	I		VIII		X		XV	
PHENYLALANINE INTAKE, *mg/kg/day*	183		0		193		0	
AVERAGE WEIGHT, *kg*	3.75		4.69		5.30		5.73	
URINE AMINO ACIDS, *per day*	*mg*	*μmole*	*mg*	*μmole*	*mg*	*μmole*	*mg*	*μmole*
Alanine	5.3	59.5	13.1	147.0	6.0	67.4	8.2	92.1
Arginine	1.6	9.2	3.2	18.4	0	0	0	0
Aspartic acid	4.4	33.1	9.1	68.4	3.4	25.7	5.4	40.6
Cystine	0.8	3.3	4.9	20.4	1.8	7.4	1.4	5.8
Glutamic acid	8.3	56.5	20.7	141.0	9.7	65.9	5.3	36.1
Glycine	25.2	336.0	62.1	827.0	12.6	167.4	27.0	360.0
Histidine	7.0	45.1	37.8	243.5	15.1	97.2	27.3	176.0
Isoleucine	2.4	18.3	2.4	18.3	3.2	24.4	3.8	29.0
Leucine	2.3	17.5	2.2	16.8	1.6	11.9	1.7	13.0
Lysine complex [1]	17.0	116.1	24.4	166.8	24.1	165.0	24.6	168.0
Methionine	3.3	22.1	2.8	18.8	3.0	20.2	3.4	22.8
PHENYLALANINE	*9.0*	*54.5*	*0.8*	*4.8*	*12.7*	*76.7*	*1.5*	*9.1*
Proline	7.1	61.7	17.1	148.5	Trace	Trace	0	0
Serine (includes asparagine and glutamine)	20.9	199.0	22.9	218.0	12.5	119.0	24.6	234.0
Threonine	6.7	56.3	15.2	127.8	7.7	64.5	9.7	81.5
Tryptophan	1.8	8.8	3.4	16.7	0	0	2.3	11.3
Tyrosine	2.4	13.2	4.3	23.8	2.5	13.7	4.5	24.9
Valine	1.8	15.4	3.1	26.4	2.1	18.0	3.2	27.3
Total identified amino acids	127.3	1125.6	249.5	2252.4	117.9	944.4	153.9	1331.5
Urine volume, ml/day	237		468		554		583	
Urine total N, gm/day	0.547		1.82		1.39		1.95	
Free (identified on column) amino N (mg/day)	15.8		31.5		13.2		18.7	
(% total N)	2.9		1.72		0.95		0.96	
Free amino N (Van Slyke) (mg/day)	22.6		40.6		20.0		27.5	
(% total N)	4.2		2.23		1.4		1.4	

[1] This may include small and variable amounts of other amino acids e.g. 1-methyl histidine and ornithine.

9.0 to 0.8 mg per day and the other a decrease from 12.7 to 1.5 mg per day. These observations, like those on threonine, indicate a close relationship between the specific amino acid deficiency and the excretion of this particular amino acid in the urine. The increased free aminoaciduria, mentioned above, which was observed in the phenylalanine-free period is apparently due to several different amino acids. Glycine excretion is increased, as might be expected from the increased glycine intake, since glycine was substituted for phenylalanine in the diet. As was the case in threonine deficiency the excretion of histidine was increased, but here even more so than in the threonine-deficiency studies. Of questionable significance were slight increases in the excretion of alanine and of aspartic acid.

The question may be raised whether the requirement of an amino acid, as determined by means of an amino acid diet, is similar to that on a diet of unsplit protein. It is conceivable that the process of hydrolysis in the intestine under the influence of the digestive enzymes presents amino acids for absorption in a ratio more favorable for protein synthesis and other physiological purposes. If presented in a less favorable ratio, as might be the case when an amino acid mixture is fed there might occur increased spillage of some amino acids in the urine. The less perfect utilization would then result in an unduly high figure for the requirement.

With this possibility in mind we have made comparisons of the loss of amino acids in the urine on milk proteins and corresponding amino acid mixtures. Some differences were encountered but the order of magnitude was so small that they did not introduce an error into the figure for the amino acid requirement.

Of interest in this connection are figures for the daily phenylalanine intake from the data, in part unpublished, of Swanson ('32) who fed a normal infant pooled breast milk for the first 4½ months of life. The nitrogen intake was accurately measured. Using data from Macy et al. ('53) for the amino acid composition of breast milk it appears that this infant

ingested from 87 to 131 mg of phenylalanine per kilogram per day with an average of 116 mg. During this period the infant remained healthy and made excellent progress.

SUMMARY

A study to evaluate the phenylalanine requirement of infants was carried out employing a synthetic diet, the nitrogen moiety of which consisted of a mixture of 18 amino acids, essential and non-essential, all as the natural L-isomers. By graded reduction of the phenylalanine, which was replaced by glycine, the minimal phenylalanine intake compatible with normal health was determined. It was found to be approximately 90 mg per kilogram per day.

A deficiency of phenylalanine was associated with failure to gain weight, impaired nitrogen retention, due primarily to increased azoturia and hypoglobulinemia. An increase in free amino acid excretion in the urine was observed. The excretion pattern of free amino acids in the urine showed a striking decrease in phenylalanine itself and certain other consistent changes, notably an increased histidinuria.

ACKNOWLEDGEMENTS

We wish to express our indebtedness to Eileen Hasselmyer, R. N. for her careful nursing supervision of these patients and to Miriam Katz, R. N.; to A. L. McDowell who performed the nitrogen determinations and to Dr. Hilda Wiese for technical advice.

LITERATURE CITED

MACY, I. G., H. KELLY AND R. SLOAN 1953 The composition of milks. Bulletin of the National Research Council no. 254.

PRATT, E. L., S. E. SNYDERMAN, M. W. CHEUNG, P. NORTON, L. E. HOLT, JR., A. E. HANSEN AND T. C. PANOS 1955 The threonine requirement of the normal infant. J. Nutrition, *56:* 231.

SWANSON, W. W. 1932 The composition of growth. II. The full term infant. Am. J. Dis. Child., *43:* 10.

A COMPARISON OF TITANIC OXIDE AND CHROMIC OXIDE AS INDEX MATERIALS FOR DETERMINING APPARENT DIGESTIBILITY

L. E. LLOYD, B. ELAINE RUTHERFORD AND E. W. CRAMPTON
Department of Nutrition, Macdonald College, McGill University, Quebec, Canada

(Received for publication December 8, 1954)

In recent years, the use of index materials has attained considerable importance in facilitating the determination of apparent digestibility. Chromic oxide (Cr_2O_3), the most common of such materials, has been used successfully to determine apparent digestibility in all monogastric species thus far tested. However, in the case of ruminants, Cr_2O_3 is not an ideal index substance as demonstrated by the results of Barnicoat ('45) and Crampton and Lloyd ('51) with sheep, and because a diurnal variation in the excretion of Cr_2O_3 by cattle was noted by Hardison and Reid ('53).

After a lapse of several years, attention has been refocussed on titanic oxide (TiO_2) by a group of French investigators when they used this substance in absorption studies with rats. Fournier ('50) used TiO_2 to determine the site of calcium absorption from the gastro-intestinal tract, and Fournier et al. ('53) followed up this work by using the index material to determine the site of phosphorus absorption. This is of interest since much earlier Lehmann and Herget ('27) had found in experiments with different species that the ingestion of large amounts of TiO_2 did not result in the deposition of this substance in the tissues of animals, and Askew ('31–'32) actually suggested the use of TiO_2 as an index material in digestibility studies with sheep. However, no reference to the use of TiO_2 in digestibility studies has been found in the literature since that time.

The purpose of this experiment was to test the value of TiO_2 as an index material for determining the apparent digestibility of a rat diet, and to compare the relative usefulness of TiO_2 and Cr_2O_3 as index substances.

EXPERIMENTAL

Since titanic oxide has not been used previously in digestibility studies, the time required for its maximum and constant excretion in the feces had to be determined first. This was followed by a comparison of the dry matter digestion coefficients calculated by the TiO_2 and "time collection" methods, and of the relative usefulness of titanic oxide and chromic oxide as index materials.

To this end, 60 male albino rats, averaging 60 days of age at the beginning of the test, were maintained in individual cages throughout the experimental period. All rats consumed a diet consisting of 90% ground wheat plus 10% of a suitable protein-mineral supplement. This diet as fed to half the animals contained 0.25% TiO_2, while for the remaining 30 rats it contained 0.25% Cr_2O_3.

From 10 of the 30 rats receiving the TiO_2-containing diet, total feces were collected individually and daily for 13 days following the initial consumption of the index material. Individual food consumption records for these rats were kept only for the final 7 days of the 13-day period. The remaining 20 rats in this group were divided into 4 lots of 5 animals each, and after a 6-day preliminary feeding period, total feces were collected and composited for each lot for a period of 7 days. Total food consumption for each lot was noted for the 7-day period.

The 30 rats receiving the Cr_2O_3-containing diet were divided into 6 lots of 5 animals each, and after a 6-day preliminary feeding period, total feces were collected and composited for each lot for a period of 7 days. Total food consumption for each lot again was determined for the latter period only.

All feces samples, depending on the origin of the residual material, were analyzed for Cr_2O_3 by the method of Bolin et al. ('52), or for TiO_2 by a method to be described.

The standard determination of TiO_2, originating with Weller (1882), depends upon the fact that acid solutions of titanium sulphate are coloured yellow to orange when treated with hydrogen peroxide. Weller's method was altered in that a modification of the wet-ash technique of Bolin et al. ('52) was applied to obtain the acid solution of titanium sulfate.

Preparation of oxidizing reagent: to 150 ml of distilled H_2O, add slowly 150 ml of concentrated H_2SO_4 and cool. Add 200 ml of $HClO_4$ (70 to 72%), and mix thoroughly.

Place a 200 to 500 mg sample of food or feces in a micro Kjeldahl digestion flask calibrated at 110 ml. Add 5 ml of oxidizing reagent to the flask and heat, gently at first, until digestion is completed. Add an additional 5 ml of conc. H_2SO_4 and heat to boiling until a clear, colourless solution is obtained. Cool, and add 2 ml of 30% H_2O_2. After 20 minutes dilute to 110 ml with distilled H_2O. Measure light transmission with a photoelectric colorimeter at 440 mμ, using distilled H_2O as a blank. Determine the amount of TiO_2 from a calibration curve in which milligrams of TiO_2 (0 to 10 mg) are plotted against optical density readings.

To test the applicability of the method, known amounts of TiO_2 were added to samples of feed and feces, and determined by the method described above. Complete recovery of the TiO_2 was obtained in all cases.

RESULTS

The apparent digestibility of dry matter, calculated by the TiO_2 ratio method on daily collections of feces for the first 6 days after the inclusion of TiO_2 in the diet, are given for 10 rats in table 1. Shown as well are dry matter digestion coefficients calculated by the TiO_2 method on feces originating from the same rats, but collected and composited from the 7th to 13th day after the dietary inclusion of TiO_2.

It is obvious from the mean values shown in table 1 that a constant and maximum excretion of TiO_2 in the feces had not been attained even after a preliminary feeding period of 6 days. This is in contrast to results obtained in this laboratory during the past 4 years in the cases where Cr_2O_3 was used as the index material in rat digestibility studies. A constant and maximum excretion of Cr_2O_3 has always been obtained after a preliminary feeding period of three to 4 days, and a

TABLE 1

The apparent digestibility of dry matter calculated by the TiO_2 ratio method on feces samples collected at different time intervals following the dietary inclusion of TiO_2

RAT NO.	APPARENT DIGESTION COEFFICIENTS FOR DRY MATTER						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th to 13th days
	%	%	%	%	%	%	%
61	69	80	81	80	80	80	82
62	63	82	82	84	81	83	82
63	72	79	80	83	81	80	82
64	65	79	81	83	83	83	83
65	52	80	81	80	83	83	84
66	66	82	82	82	82	83	85
67	67	78	80	82	81	82	84
68	72	83	81	81	83	84	85
69	68	80	78	77	79	81	83
70	72	79	77	81	82	81	80
Mean	67	80	80	81	82	82	83

complete daily recovery of the index has been possible after that time.

The apparent digestion coefficients of dry matter for all rats, determined by (1) either the TiO_2 or Cr_2O_3 ratio method using the 7th to 13th day composited feces, and (2) the conventional "time collection" method for the same period, are found in table 2.

It is of importance to note that in all instances, that is, for individual rats and for lots of 5 rats each, the apparent digestion coefficients calculated by the titanic oxide method were lower than those determined by the "time collection"

method. On the other hand, for 5 of the 6 lots of rats receiving chromic oxide in the diet, the apparent digestibility of dry matter calculated by the ratio method was identical to that determined conventionally. For the other lot, the digestion coefficient was actually one percentage unit greater by the Cr_2O_3 method.

TABLE 2

The apparent digestibility of dry matter calculated by either of the index methods and by the convention "time collection" method on feces samples excreted and composited from the 7th to 13th day following the dietary inclusion of index material

RAT NO.	INDEX METHOD USED	APPARENT DIGESTION COEFFICIENTS FOR DRY MATTER	
		Index method	"Time collection" method
		%	%
61	TiO_2	82	85
62	TiO_2	82	83
63	TiO_2	82	84
64	TiO_2	83	85
65	TiO_2	84	85
66	TiO_2	85	86
67	TiO_2	84	85
68	TiO_2	85	87
69	TiO_2	83	84
70	TiO_2	80	84
71– 75	TiO_2	82	84
76– 80	TiO_2	84	85
81– 85	TiO_2	84	85
86– 90	TiO_2	84	85
91– 95	Cr_2O_3	85	85
96–100	Cr_2O_3	84	84
101–105	Cr_2O_3	85	85
106–110	Cr_2O_3	85	85
111–115	Cr_2O_3	86	85
116–120	Cr_2O_3	85	85

The total recovery of both index materials was determined on the feces excreted from the 7th to 13th days inclusive. For the 30 rats receiving the TiO_2-containing diet, the average recovery of TiO_2 over the 7-day period was only 92.0% while for the 30 rats receiving the Cr_2O_3-containing diet, the average recovery of Cr_2O_3 was 99.8% for the same period.

DISCUSSION

One of the original objectives of this test, the determination of the length of time required for the maximum constant excretion of titanic oxide in the feces, was not realized. However, this condition had not been attained after 6 days of TiO_2 consumption, and in view of the fact that Cr_2O_3 is found in maximum amounts in the feces only three to 4 days after its inclusion in the diet, the possibility of TiO_2 serving as a more useful index material than Cr_2O_3 becomes remote. The low apparent digestion coefficients for dry matter obtained by the TiO_2 method after a preliminary feeding period of 6 days confirmed the inadequacy of TiO_2 as a suitable index substance, especially when these values were compared with those obtained by the Cr_2O_3 method. The results using Cr_2O_3 confirmed our earlier work with rats (Schürch et al., '50), and substantiated our views that Cr_2O_3 is the most useful of the index materials thus far tested, at least for the species under study, and probably for all monogastric species.

The low digestion coefficients obtained with TiO_2, a reflection of the observed incomplete recovery of this material in the feces, are difficult to explain at this time. That titanic oxide was absorbed from the gastro-intestinal tract seems unlikely, since with progressing time, the average digestion coefficients approached the values determined conventionally. A delayed excretion of TiO_2 due to its accumulation in some part of the tract, possibly in the caecum, seems a more likely possibility. This suggestion is made in spite of (1) the fact that TiO_2 has a slightly lower specific gravity than Cr_2O_3, and (2) the observation made by Fournier ('50) that after 6 hours of fasting, the stomach of the rat contained only a trace, and after 24 hours, neither the stomach nor the intestine contained a trace of TiO_2.

Therefore, the reasons for the failure of TiO_2 to serve as an adequate index material in digestibility studies with rats must await further research, but the fact that Cr_2O_3 is superior to TiO_2 for this purpose has been established.

CONCLUSIONS

When compared to chromic oxide, titanic oxide was found to be inadequate as an index material for determining the apparent digestibility of a rat diet.

LITERATURE CITED

ASKEW, H. O. 1931 Note on effect of feeding titanium oxide to sheep. New Zealand J. Sci. and Technol., *13:* 76.

BARNICOAT, C. R. 1945 Estimation of apparent digestibility coefficients by means of an inert "reference substance". New Zealand J. Sci. and Technol., *27:* 202.

BOLIN, D. W., R. P. KING AND E. W. KLOSTERMAN 1952 A simplified method for the determination of chromic oxide (Cr_2O_3) when used as an index substance. Science, *116:* 634.

CRAMPTON, E. W., AND L. E. LLOYD 1951 Studies with sheep on the use of chromic oxide as an index of digestibility of ruminant rations. J. *Nutrition, 45: 319.*

FOURNIER, P. 1950 De l'emploi de l'oxyde de titane pour l'étude quantitative de l'absorption intestinale. Compt. Rend. Acad. Sci. (Paris), *231* (23): 1343.

FOURNIER, P., AND Y. DUPUIS (WITH A. BOURDEAU) 1953 An indirect method of estimating the absorption of phosphorus compounds from different parts of the digestive tract. J. Physiol. (Paris), *45:* 443.

HARDISON, W. A., AND J. T. REID 1953 Use of indicators in the measurement of the dry matter intake of grazing animals. J. Nutrition, *51:* 35.

LEHMANN, K. B., AND L. HERGET 1927 Studien über die hygienischen Eigenschaften des Titandioxyds und des Titanweiss. Chem. Ztg., *51:* 793.

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.

WELLER, A. 1882 Colorimetric determination of titanium. Ber., *15:* 2593. Found in Treadwell and Hall's analytical Chemistry, Vol. II Quantitative, 1942.

EXPERIMENTS WITH INTERMITTENT FEEDING OF PROTEIN TO RATS [1]

E. GEIGER, IHSAN EL RAWI [2] AND H. V. THOMAS
Department of Pharmacology, University of Southern California Medical School, Los Angeles, and Van Camp Seafood Co., Terminal Island, California

THREE FIGURES

(Received for publication December 3, 1954)

The protein requirement of man and of different animals is usually defined as the quantity which must be consumed during a 24-hour period in order to promote growth or maintain good health. Such a definition serves equally well for scientific or dietetic purposes and can be used with advantage in those cases where calculated quantities of foods can be provided each day. However, such a definition does not take into consideration practical human nutrition where the day to day protein intake may vary considerably. For instance, in many countries, people who work in the fields or woods far from home during weekdays, consume only small quantities of protein while at work but on the week ends eat meals providing much larger amounts of protein. Such people usually show no signs of protein deficiency in spite of the fact that the 24-hour requirements are not provided regularly. It has even been claimed that people with limited food supply who eat their high quality foods during two or three days are in better general health than those who consume similar rations equally divided during the whole week (Volkmann, '47). These observations suggest that dietary protein may be better utilized during the

[1] Supported by a grant from the Alaskan Air Command, Arctic Acromedical Laboratory, Ladd AFB, Alaska (Contract no. AS189600)-549, and the Williams Waterman Fund of the Research Corp.

[2] Presently with FAO in Djakarta, Indonesia.

days immediately following periods of restricted protein intake. This assumption is further supported by experiments in which it has been demonstrated that following a period of protein deficiency, nitrogen retention, i.e., positive nitrogen balance, can be induced by the feeding of relatively small amounts of protein.[3] The results of such experiments, however, are not convincing since the fundamental experiments of Allison ('51) have shown that nitrogen retention depends on many other factors and therefore positive nitrogen balance by itself is not an adequate measure of protein utilization for building of body substance.

We planned, therefore, to investigate the question of whether and by what means the utilization of dietary protein is changed by a prior period of protein restriction; whether there is a metabolic adaptation to the curtailed protein supply. In the last few years two pertinent papers have been published. One of them, by Harte, Travers and Sarich ('48) showed that "weanling rats grow as well and utilize dietary protein with the same efficiency for growth, whether it is supplied at a constant level in the diet or whether the level is alternated daily about the same value as a mean . . ." In these experiments, however, protein was withheld for only one day and this period may have been too short to influence protein utilization. In the other series of experiments (Gernand, '51), it was shown that rats kept on low-caloric diets lost weight constantly and succumbed within 6 to 15 weeks. Another group of animals, however, which received the same total amount of food during each 8-day period, but which was fed the fat and protein allowances only during the 6th, 7th, and 8th days following 5 fat- and protein-free days, gained constantly and survived in good condition. Gernand ('51) therefore concluded that periodic ("stossweise") feeding of protein and fat is more efficient than evenly divided daily feeding of the same quantities.

We planned to use these results of Gernand as a basis for further analysis of the factors which may influence the utiliza-

[3] For literature see B. Bray, '53.

tion of dietary proteins. However, preliminary experiments, in which we tried to reproduce the work of this author, led to unexpected results which necessitated a re-investigation of the whole problem

METHOD

Male, white rats of the Wistar strain were caged individually and kept in a temperature-controlled (21°C.) room. The

TABLE 1

Percentage composition of diets

COMPONENT	EXPERIMENT 1			EXPERIMENT 2			EXPERIMENT 3		
	A *gm*	B *gm*	C *gm*	D *gm*	E *gm*	F *gm*	G *gm*	H *gm*	I *gm*
Cornstarch	65	97	40.9	58.3	80.4	39.2	61	69	55
Cottonseed oil	10		17.7	12.2	16.6	8.4			
Lactalbumin	22		38.4	26.5		49.4			
Casein							11.82	3.94	17.73
DL-Methionine							0.18	0.06	0.27
Ruffex [1]							5.00	5.00	5.00
Lard							9.00	9.00	9.00
Salt mixture, U.S.P. no. 2	3	3	3	3	3	3	5	5	5
Wheat germ oil							4	4	4
Cod liver oil							4	4	4
Vitamin mixture [2]									

[1] Powdered and extracted rice bran hull supplied by Fisher Scientific Company, Pittsburgh.

[2] Vitamins supplemented per 1,000 gm of each of the diets: A, 10,000 U.S.P. Units; D, 2,000 U.S.P. Units; thiamine, 4 mg; riboflavin, 4 mg; pyridoxine HCl, 0.2 mg; calcium pantothenate, 12 mg; niacin amide, 40 mg; folic acid, 5 mg; biotin, 2 mg; B_{12}, 10 μg; choline chloride, 4.2 gm.

food was offered in double cups which made it possible to determine the food consumption with fair accuracy.

The composition of the diets is described in table 1. In experiment 1 the feeding plan described by Gernand was followed. The rats of group A were offered 5.9 gm of complete diet A daily; group B received 4 gm of a fat- and protein-free diet, B, during the first 5 days. However, on the 6th, 7th, and 8th days, this group was fed 9.1 gm of diet C. The diets con-

tained amounts of protein and fat calculated so that the total food intake for an 8-day period was the same in both groups.

A similar plan, using diets D, E, and F, was followed in experiment 2. In this experiment, however, the daily diets of both groups contained equal amounts of carbohydrate and fat, and only the protein moiety of the diet was fed intermittently.

In experiment 3, which was performed on both adult and young rats, the control group received diet G (12% casein), and the experimental group received alternately diets H and I, with protein contents of 4 and 18% respectively.

In experiments 1 and 2 weighed amounts of diet were offered and consumed by the rats. The body weights were determined weekly. In experiment 3 the animals received food ad libitum, and the quantities consumed, as well as the body weights, were determined daily.

In experiments 1 and 2, and in part I of experiment 3, young rats of an average body weight of 80 gm were used. In part II of experiment 3, however, the maintenance of body weight was studied in adult animals weighing an average of 410 gm.

RESULTS

In experiment 1 the problem of whether intermittent feeding of fat and protein improves food utilization, as claimed by the above quoted authors, was investigated. In these experiments the rats of group A (20 animals) received daily 5.9 gm of complete diet A, containing 26.5 Cal. The rats in group B (20 animals) received for 5 days 4 gm of the carbohydrate diet, B, providing 16 Cal., and on the 6th, 7th, and 8th days of each period were given 9.1 gm of diet C which contained 44.4 Cal., and protein and fat in such quantities that the total amount fed on these three days equalled the quantity consumed by the rats of group A during the total 8-day period. These quantities of diets were fed for 32 days, i.e., for 4 8-day periods. During this time the animals of both groups gained weight, but the increase in group B with intermittent feeding was less than that in group A with equal distribution of

dietary protein and fat. After 32 days the intake of all three diets was reduced by 25%. On this regime, the rats lost weight similarly in both groups (fig. 1). The results of experiment 1 indicate, therefore, that periodic feeding of the fat and protein fraction of the diets does not improve their utilization.

For experiment 2 the diets were modified so that only the protein was fed intermittently, whereas the dietary carbohydrate and fat were evenly distributed. The total caloric fat and protein intake for the 8-day period was the same for

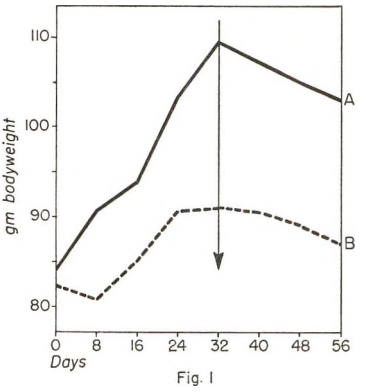


Fig. 1

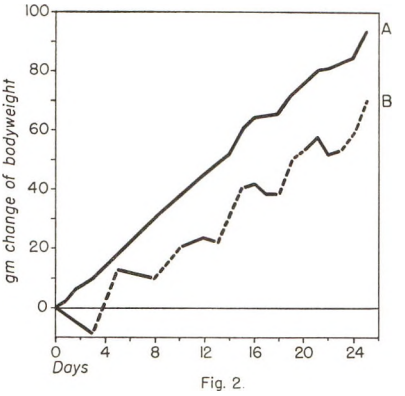


Fig. 2.

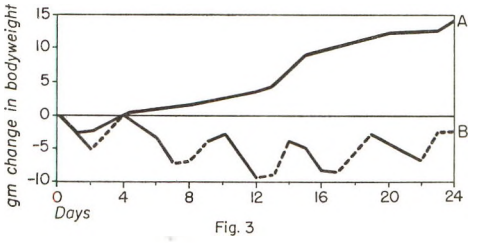


Fig. 3

Fig. 1 Body weight of rats on (A) even; (B) intermittent protein and fat feeding. At arrow decrease of caloric intake. (Experiment 1.)

Fig. 2 Change of body weight of young rats (A) on even; (B) on intermittent protein feeding. (Experiment 3.)

In curve B ———— low protein diet; – – – – high protein diet.

Fig. 3 Change of body weight of adult rats (experiment 3). (A) on even; (B) on intermittent protein feeding.

In curve B ———— low protein diet; – – – – high protein diet.

both groups. The result of this experiment was similar to that of experiment 1. The rats receiving the complete diet containing 22 Cal. daily grew better than those receiving protein only on the 6th, 7th, and 8th days. In group A (10 rats) the average growth for the 32-day period was 18 ± 2.4 gm, and for group B, receiving protein only on the 6th, 7th, and 8th days, the corresponding average growth was — 10 ± 3.5 gm.

Experiment 2, like experiment 1, shows that rats grow better when restricted amounts of dietary protein are evenly distributed during the 8-day period. Therefore it is concluded that alternating the feeding of protein- and fat-free diets with diets containing high concentrations of protein and fat is less effective than feeding the protein in equally divided daily portions even though the total intake for the 8-day period is the same.

In experiment 3 the effect on protein utilization of intermittent feeding of a calorically satisfactory diet was investigated, using a different feeding schedule and food composition. In addition to the above question, the problem of whether, during the period of insufficient protein intake a "protein debt" develops for which the animals compensate by increased intake of protein when such is made available was investigated.

For experiment 3 the diets were designed to approximate actual conditions more closely than in experiments 1 and 2. Because most natural food contains small amounts of protein, days with high protein intake usually alternate with those of low protein consumption rather than with protein-free days. In experiment 3, therefore, diet H, with a low protein content, was offered to the rats instead of protein-free fod.

In this experiment the animals received food ad libitum. The food consumption and body weights were determined daily. For this experiment shorter periods of protein restriction were used since preliminary experiments confirmed the results reported by Jackson ('37), namely that the voluntary

TABLE 2

Changes in body weight and food consumption of rats on even and intermittent feeding of protein

(Experiment 3)

PLAN OF FEEDING	DAILY FOOD INTAKE IN GRAMS			PROTEIN CONSUMPTION IN GRAMS WITH			TOTAL PROTEIN CONSUMED	CHANGE IN BODY WEIGHT	GAIN IN BODY WT. PER GM PROTEIN CONSUMED
	Diet I [3]	Diet G [1]	Diet H [2]	Diet G	Diet H	Diet I			
				I. Young rats					
A [4] (8) [5]	14 ± 4.4			41			41	+ 93 ± 5.1	2.26
B [6] (8)		10.8 ± 1	14.3 ± 0.6		6.48	25.8	32.3	+ 70 ± 3.3	2.19
				II. Adult rats					
A [4] (7)	14.9 ± 0.9			42.9			42.9	+ 12.4 ± 4.1	...
B [6] (8)		14.3 ± 0.6	14.3 ± 0.28		8.6	26.3	34.9	— 2.4 ± 3.1	...

[1] For 25 days.
[2] For 15 days.
[3] For 10 days.
[4] Even distribution of protein feeding.
[5] Number of rats used is given within parentheses.
[6] Intermittent feeding of low- and high-protein diets.

food intake decreases only slightly during the first three days after the animals are put on a low-protein diet. The results of this experiment are condensed in figure 3 and table 2.

In part I of experiment 3 (table 2) young growing rats were used. In the second part, the maintenance of adult animals was studied. The control groups in both parts of experiment 3 were given free access to diet G, containing 12% protein, for the duration of the experiment, 25 days. The young control rats of this group demonstrated a steady gain in body weight. The animals in group B received diet H containing 4% protein during three days of each 5-day period, and on the 4th and 5th days of this period diet I containing 18% casein.

The young rats in this group lost weight on the days on which 4% protein was fed. On the two consecutive days with 18% protein intake, a rapid growth occurred, but in spite of this the animals of this group did not reach the weight of those of the control group.

An analysis of the food intake indicates that the animals of the control group consumed 350 gm of food providing 1,552 Cal. and 42 gm of protein during the 25-day experiment. The rats of group B, however, consumed 161 gm of food with 716 Cal. and 6.5 gm of protein during the 15 days on a low-protein diet; during the 10 days on the high-protein diet I, 143 gm of food providing 636 Cal. and 25.8 gm of protein had been eaten. Therefore, the total intake during the 25-day period for this group was 1,352 Cal. and 32.3 gm of protein. This means that during the whole experiment the animals on intermittent feeding consumed, on the average, 200 Cal. less than those of the control group. The protein intake was also 9.7 gm less. The total growth calculated per gram of protein intake was 2.28 for group A and for group B, 2.19 gm, i.e., the utilization of dietary protein for body growth was practically the same in the two groups.

In these experiments, then, young animals receiving high- and low-protein diets alternately grew less well than the control animals, probably because of the somewhat decreased

food intake. The results on adult animals were similar for the most part. The animals (fig. 3) with an initial weight of 400 gm gained, on even feeding, 12.4 gm, while those on intermittent feeding lost on the average 2 gm of their original body weight. A calculation of the data presented in table 2 shows again that the total protein intake for the group fed intermittently was 8.5 gm less than that in the other group.

DISCUSSION

These experiments do not confirm those of Gernand ('51) who found that intermittent feeding of protein improves growth and nutritional condition of the animals on restricted diets. The reason for this contradiction is probably the difference in composition of the diets used. Detailed quantitative data concerning the diet were not published by the author in his original paper, but Doctor Gernand has been kind enough to provide this information in a private communication.[4] The diet used by Gernand does not seem to be of satisfactory composition because his animals succumbed when fed an amount equivalent to 26 Cal., while our diet at this caloric level definitely promoted the growth of animals of similar weight. The diets he used do not conform to our currently recognized nutritional standards. It is easily possible that the absence of some vitamins or the amino acid imbalance created by the large amounts of gelatin (Krehl et al., '46) may have created specific conditions which are reflected in the author's results. A further possible reason for the differences may be that Gernand's rats were of a different strain than ours.

The results of experiment 3 with ad libitum feeding show that rats receiving diets with high and low protein content alternately do not grow as well as the animals which are fed

[4] Composition of the diets used in Gernand's experiments:

I.	Sugar	200 gm	II.	Dry meat	40 gm
	Rye flour	300 gm		Meat-flour	40 gm
	Cellulose	60 gm		Salt mixture	6 gm
	Salt mixture	28 gm		Defatted wheat germ	100 gm
	Water	1580 gm		10% gelatin solution	300 gm
				Cod liver oil	25 gm
				Butter	30 gm

A mixture of the two diets was fed in the control experiment.

daily diets containing moderate but satisfactory concentrations of protein. The main reason for this difference seems to be that the total protein intake of rats on ad libitum alternating feeding is smaller than that of the control animals.

It is important to note that during the period on low protein diets the rats did not consume larger amounts of the diet in order to compensate for its low protein content. Furthermore, when protein-rich diets were offered after three days on low-protein feeding, the food intake did not increase and was nearly identical with that of the control animals. These results indicate that protein restriction in rats does not create a specific protein hunger which leads "instinctively" to increased protein intake when protein-rich diets are made available. Therefore, these results suggest as well that the daily food intake is determined primarily by the caloric value of the food consumed and not by such specific factors as protein requirement.[5]

The food consumptions during the low-protein period differed in the two groups of experiment 3. In the young growing rats the food consumption decreased progressively. It dropped on the first day from the average of 14 gm to 12, on the second day to 10, and on the third to about 9. Such decrease in food intake on low-protein diets has been observed by several authors (Frazier et al., '47; Rose, '38) and seems to be the consequence of a metabolic disturbance due to protein deficiency. Adult animals seem to be more resistant to short-term protein deprivation and therefore their food intake did not decrease during the three-day period in experiment 3.

All of the experiments reported here indicate that diets containing normal amounts of proteins and offered daily in equally divided doses promote growth and maintenance better than diets which supply in alternation low and high amounts of protein.

Therefore these experiments seem to confirm our previous report (Geiger, '51) that optimum utilization of protein is

[5] For pertinent bibliography, see Lepkovsky, '48.

obtained when the rate of intake closely parallels the growth or maintenance requirements of the body.

SUMMARY

1. Experiments with rats on low-caloric intake show that periodic feeding of the protein fraction does not improve the utilization. The rate of growth on such intermittent feeding is lower, even though the total protein intake for the 8-day period is the same as in the control experiments in which the protein quota was fed in equally divided daily doses.

2. Experiments with ad libitum feeding indicate that diets containing 14% of protein and offered daily promote growth and maintenance better than alternation between protein-rich (18%) and protein-low (4%) diets.

3. Short-term protein restriction in rats does not seem to create a specific protein hunger which would "instinctively" increase the protein intake when protein-rich diets are made available.

4. The experiments support the view that growth and maintenance are at an optimum when the rate of protein intake closely parallels the growth and maintenance requirements of the body.

LITERATURE CITED

ALLISON, J. B. 1951 Interpretation of nitrogen balance data. Fed. Proc., *10:* 676.

BRAY, B. 1953 N-metabolism in West African children. Brit. J. Nutrition, *7:* 10.

FRAZIER, L. E., R. W. WISSLER, C. H. STEFFEE, R. L. WOOLRIDGE AND P. R. CANNON 1947 Studies in amino acid utilization. J. Nutrition, *33:* 65.

GEIGER, E. 1951 Extra caloric function of dietary components. Fed. Proc., *10:* 670.

GERNAND, K. 1951 Stossweise Fett-Eiweisszufuhr bei gleichzeitiger calorischer Unterernaehrung. Klinische Wchschft., *30:* 199.

HARTE, R. A., J. J. TRAVERS AND P. SARICH 1948 Protein alternation and rat growth. J. Nutrition, *35:* 287.

JACKSON, C. M. 1937 Food intake with protein deficiency. Ibid., *13:* 669.

KREHL, W. A., P. S. SARMA AND C. A. ELVEHJEM 1946 Niacin and tryptophane requirement. J. Biol. Chem., *162:* 403.

LEPKOVSKY, S. 1948 Physiological basis of voluntary food intake. Adv. in Food Res., *1:* 105.

ROSE, W. C. 1938 The nutritional significance of amino acids. Physiol. Rev., *18:* 109.

VOLKMANN, J. 1947 Letter to the Editor. Deutches Gesundheitwesen, *17:* 545.

VITAMIN D AND MAGNESIUM ABSORPTION [1]

ROGER B. MEINTZER AND HARRY STEENBOCK
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication December 17, 1954)

The effect of vitamin D on the absorption of Mg is of more than passing interest, for while Day and McCollum ('39) found that 85% of ingested Mg was absorbed by rats on a low P ration, it has been assumed generally that it is poorly absorbed. (Mendel and Benedict, '09; Schmidt and Greenberg, '35; Tibbetts and Aub, '37; Leichsenring et al., '51.) This conclusion was based on results obtained with other animals than the rat and with other than purified diets. The role of vitamin D cannot be assessed from the published data. In the experiments of Day and McCollum vitamin D was fed to all of the rats used in their experiments. Outside of the data obtained in metabolic trials the published data on the Mg content of tissues also are not helpful in determining the effect of vitamin D. While the Mg content of rat bones has been found to be increased in rickets (Gassmann, '10; von Euler and Rydbom, '31) the amount in the blood serum apparently is reduced (Bomskov and Kruger, '31).

Our interest in the effect of vitamin D on Mg absorption originated through our concern over the possible modifying effect of changes in Mg absorption on Ca and P balances when vitamin D was given. An ionic antagonistic effect of Ca and Mg is well recognized and as some of our observed effects of vitamin D were not those ordinarily attributed to this vitamin

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison. This research was supported by funds provided by the Wisconsin Alumni Research Foundation.

we have sought to ascertain if the Mg content of the diet could be a factor. On the supposition that differences in the published reports of the availability of Mg might have been arrived at with rations differing in P content or in the nature of the P in the rations some experiments were carried out with semi-synthetic rations in which P was added as inorganic phosphate or as phytic acid under acidic as well as basic conditions.

EXPERIMENTAL METHODS

Ninety-gram rats of the Sprague-Dawley strain were placed in metabolism cages for a 12-day period, during the last 9 days of which urine and feces were collected. Daily, for three or more half-hour periods, the rats were transferred for feeding to similar cages provided with sub-bottoms of filter paper to absorb any urine voided and to catch feces. At the end of the balance period the feces excreted in these cages were added to those collected in the metabolism cages and the filter papers, after brushing off any spilled food, were eluted with hot normal hydrochloric acid and rinsed twice with hot water. These elutions and rinses were added to the urine collections.

The food consumption was limited to 20 gm per rat for each three-day period in such a manner that this allotment lasted throughout the period and yet was consumed completely. This amount supported only very limited growth but this equalization of food intake eliminated difficulties due to variations in appetite and in the resultant mineral intake.

The basal ration was a modification of the semi-synthetic diet of Steenbock et al. ('51). It was a low P (0.016%), low Ca (0.012%), low Mg (0.02%) ration consisting of Cerelose (D-glucose monohydrate) 67, cooked egg white 18, roughage [2] 3, cottonseed oil [3] 10, and Ca-P-Mg-free salts 2. In this ration the level of magnesium has been reported to supply 4 times

[2] Ruffex.
[3] Wesson.

the minimum needs of the rat for growth, reproduction and lactation (Schmidt and Greenberg, '35).

Crystalline vitamins were added to the ration in a mixture with glucose. One gram of the mixture per kilogram of ration provided the following number of milligrams of the respective vitamins; thiamine hydrochloride, 4; riboflavin, 5; pyridoxine, 5; calcium pantothenate, 28; nicotinamide, 10; p-aminobenzoic acid, 200; and inositol, 200. Choline chloride was added to the ration at a level of 500 mg per kilogram of ration as an alcoholic solution sprayed on the Cerelose before the ration was mixed.

A supplement of 70 μg of β-carotene, 105 μg of 2-methyl-1,4-naphthoquinone, and 875 μg of α-tocopherol in cottonseed oil was supplied each week in three doses. Vitamin D, when given, was administered orally at the rate of 75 I.U. per three-day period as crystalline calciferol dissolved in cottonseed oil.

The salt mixture used by Steenbock et al., ('51) was modified by the omission of the Mg ingredient. Mineral additions to the basal diet were made at the expense of the Cerelose. Magnesium phosphate of the desired Mg/P ratio was prepared by dissolving $MgCO_3$ in standardized phosphoric acid, drying and grinding. The Mg phytate was prepared from a technical calcium phytate preparation [4] by removal of the Ca and precipitation with $MgCO_3$. Two preparations of varying Mg/P ratio were made by precipitating them respectively at a pH of 5 and 8. The former, later referred to as acidic Mg phytate, contained 8.3% Mg and 12.7% P while the latter, referred to as basic Mg phytate, contained 15.2% Mg and 8.0% P. The basic preparation contained an excess of $MgC0_3$ over that which could react with the phytic acid in the solution. The amount of Mg salts which could be fed was limited by their cathartic effect. Since a level of 0.24% Mg was the maximum which could be fed, the amount included in the diet was limited to 0.12% to avoid all danger of the incidence of diarrhea. Water was supplied for ad libitum consumption.

[4] Staley.

The urines after concentration and the feces as obtained were wet-ashed with nitric acid followed by perchloric acid. Inorganic P was determined essentially according to the method of Fiske and Subba Row ('25) while phytic acid was determined by the method of Pringle and Moran ('42). Mg was determined by an analysis for P in the magnesium ammonium phosphate precipitated after the removal of Ca as the oxalate.

RESULTS

The data in table 1 reveal that vitamin D increased Mg absorption. While the increase is small, never exceeding 19% of the intake which perforce had to be limited to 7 to 8 mgs daily, it was obtained consistently in 12 separate trials entailing the use of 170 rats. There is no evidence that this increase was the result of an increase in Mg requirements due to a stimulation of growth, as growth was increased inconsistently and, when it did occur, the increase was small, undoubtedly because of the limitation in food intake. Assessed on the basis of normal Mg requirements (Schmidt and Greenberg, '35) the intake of our animals exceeded this by a multiple of 20. Furthermore, as shown in table 2, the decrease in fecal excretion was more than compensated for by an increase in the urine so that the retention of Mg was actually decreased when vitamin D was given.

It is apparent from our data that Mg was absorbed readily even when given as the phosphate or phytate or under very basic conditions such as were provided by additions of 1.0% $CaC0_3$ and 2.6% $NaHC0_3$. Obviously the retention was limited with the carbonate as the phosphorus content of the basal ration was too low to meet the requirements of tissue growth but when P was provided either as Mg phosphate or as Mg phytate retention became possible. The greatest retention occurred at the highest level of P intake, namely, at 0.34% of the ration when Mg was furnished as the phosphate.

TABLE 1

The effect of vitamin D on magnesium absorption

ADDITIONS TO THE BASAL RATION [1]	INCREASE IN WEIGHT OF RATS IN 9 DAYS		Mg ABSORBED [2]		DIFFERENCE
	− D	+ D	− D	+ D	
	gm	*gm*	%	%	%
None	13	19	67.6	69.7	2.1
$MgCO_3$	19	15	60.2 (± 2.4)	68.3 (± 2.6)	8.1
$MgCO_3$ + 1.0% $CaCO_3$ + 2.6% $NaHCO_3$	9	11	71.3 (± 3.2)	76.7 (± 2.9)	5.4
Mg phosphate	7	7	58.5 (± 4.0)	66.5 (± 3.6)	8.0
Acidic Mg phytate	7	8	52.9 (± 3.4)	62.4 (± 2.9)	9.5
Acidic Mg phytate + 1.0% $CaCO_3$	11	8	58.0 (± 9.5)	61.2 (± 1.7)	3.2
Acidic Mg phytate + 0.42% $MgCO_3$	7	12	63.4 (± 2.1)	66.4 (± 2.9)	3.0
Basic Mg phytate	12	11	59.3 (± 2.8)	68.1 (± 2.3)	8.8

[1] Basal ration was low in P (0.016%), Ca (0.012%), and Mg (0.02%). Magnesium additions were all made at a level equivalent to 0.42% $MgCO_3$.

[2] Figures in parentheses give standard error of the mean calculated as follows: $\frac{\sqrt{\frac{\Sigma d^2}{n}}}{\sqrt{n}}$ where d is the deviation from the mean and n is the number of observations. Each group consisted of 6 rats except those fed the basic Mg phytate ration which had 12.

TABLE 2

The effect of inorganic and phytic acid phosphorus on the absorption and excretion of magnesium

ADDITIONS TO BASAL RATION	ADDITION OF VITAMIN D	NO. OF RATS	DAILY INTAKE	DAILY EXCRETION [1]			DAILY BALANCE
				Feces	Urine	Total	
	25 i.u./day		*mg*	*mg*	*mg*	*mg*	*mg*
$MgCO_3$	No	3	7.58	2.92	4.27	7.19	+ 0.39
(0.016% P)	Yes	3	7.72	1.98	5.81	7.79	— 0.07
Mg phosphate	No	3	7.32	2.46	1.36	3.82	+ 3.50
(0.34% P)	Yes	3	7.41	2.09	2.30	4.39	+ 3.02
Mg phosphate	No	6	7.08	2.94 (± 0.23)	3.07 (± 0.23)	6.01 (± 0.37)	+ 1.07
(0.14% P)	Yes	6	7.20	2.41 (± 0.25)	4.62 (± 0.15)	7.03 (± 0.07)	+ 0.17
Mg phytate	No	6	8.13	3.83 (± 0.16)	3.59 (± 0.16)	7.42 (± 0.11)	+ 0.71
(0.14% P)	Yes	6	8.20	3.08 (± 0.14)	5.02 (± 0.10)	8.10 (± 0.07)	+ 0.10
Mg phytate + 1.0% $CaCO_3$	No	6	8.42	3.64 (± 0.57)	3.96 (± 0.51)	7.60 (± 0.44)	+ 0.82
(0.14% P)	Yes	6	8.06	3.13 (± 0.15)	4.68 (± 0.12)	7.81 (± 0.26)	+ 0.25

[1] The standard error of the mean is given in parentheses except where pooled samples were used.

TABLE 3

The effect of magnesium, calcium, and phytic acid on phosphorus balances

ADDITIONS TO BASAL RATION	ADDITION OF VITAMIN D	DAILY INTAKE	DAILY EXCRETION [1]			DAILY BALANCE
			Feces	Urine	Total	
	25 i.u./day	*mg*	*mg*	*mg*	*mg*	*mg*
Mg phosphate	No	9.05	2.82 (± 0.16)	2.96 (± 0.40)	5.78 (± 0.65)	+ 3.27
(0.12% Mg, 0.14% P)	Yes	9.20	1.86 (± 0.14)	4.36 (± 0.33)	6.22 (± 0.24)	+ 2.98
Mg phytate	No	9.44	4.65 (± 0.10)	2.00 (± 0.30)	6.65 (± 0.35)	+ 2.79
(0.12% Mg, 0.14% P)	Yes	9.53	3.03 (± 0.21)	3.05 (± 0.33)	6.08 (± 0.38)	+ 3.45
Mg phytate + 1.0% $CaCO_3$	No	9.60	7.41 (± 1.35)	0.09 (± 0.02)	7.50 (± 1.33)	+ 2.10
(0.12% Mg, 0.14% P)	Yes	9.19	5.06 (± 0.26)	0.20 (± 0.06)	5.26 (± 0.21)	+ 3.93
Mg phytate	No	6.58	3.64 (± 0.35)	1.11 (± 0.27)	4.75 (± 0.12)	+ 1.83
(0.12% Mg, 0.11% P)	Yes	6.89	1.47 (± 0.38)	0.59 (± 0.20)	2.06 (± 0.59)	+ 4.83
Mg phytate + $MgCO_3$	No	6.70	3.89 (± 0.27)	0.79 (± 0.23)	4.68 (± 0.17)	+ 2.02
(0.20% Mg, 0.11% P)	Yes	7.22	1.88 (± 0.42)	1.26 (± 0.27)	3.14 (± 0.34)	+ 4.08

[1] The standard error of the mean is given in parentheses. All groups consisted of 6 rats.

TABLE 4

The effect of magnesium and calcium on the hydrolysis of phytic acid

ADDITIONS TO BASAL RATION	ADDITION OF VITAMIN D	DAILY PHYTATE P INTAKE	DAILY FECAL EXCRETION [1]			PHYTATE HYDROLYSIS
			Phytate	Inorganic	Other	
	25 i.u./day	*mg*	*mg*	*mg*	*mg*	*%*
Mg phytate	No	9.44	1.25	2.50	0.51	86.7
(0.12% Mg, 0.14% P)	Yes	9.53	0.89	1.32	0.52	90.7
Mg phytate + 1.0% $CaCO_3$	No	9.60	4.82	2.39	0.57	49.8
(0.12% Mg, 0.14% P)	Yes	9.19	2.28	1.66	0.72	75.2
Mg phytate	No	6.58	0.93 (± 0.12)	1.40 (± 0.15)	1.08 (± 0.31)	85.9
(0.12% Mg, 0.11% P)	Yes	6.89	0.21 (± 0.08)	0.69 (± 0.16)	0.60 (± 0.26)	96.9
Mg phytate + $MgCO_3$	No	6.70	0.89 (± 0.11)	1.77 (± 0.14)	1.35 (± 0.34)	86.7
(0.20% Mg, 0.11% P)	Yes	7.23	0.37 (± 0.12)	0.91 (± 0.15)	0.68 (± 0.23)	94.9

[1] The standard error of the mean is given in parentheses except where pooled samples were used. All groups consisted of 6 rats.

The phosphate balances in 4 out of 5 experiments (table 3) reveal a very definite increase in P retention when vitamin D was given, the main import of which, together with the evidence on phytic acid hydrolysis (table 4) is: that phytic acid given as Mg phytate was available as a source of P in the absence of vitamin D even when its hydrolysis was depressed to 49.8% of the total intake by the addition of 1% $CaC0_3$. When fed without $CaC0_3$ the hydrolysis of added phytate was almost as great in the absence of vitamin D as when it was given. The retention of P also was essentially unchanged.

We have no explanation either for the mechanism of the increase in Mg absorption or for the decrease in its retention when vitamin D is given. Specifically the former may be attributed to an increase in the acidity of the tract or to an as yet unidentified improvement in metabolic activity in the intestinal tract as well as in other tissues. The decrease in the retention of Mg may be due to the correction of the increase in Mg content of tissue when Mg is substituted for Ca in a diet free from vitamin D. This would be in harmony with the observations of Gassmann ('10) and von Euler and Rydbom ('31) who found that the Mg content of bone is increased in rickets.

SUMMARY

A series of experiments with 90-gm rats revealed that Mg was absorbed equally well when added at a level of 0.12% of the ration as the carbonate, phosphate, or phytate to a low-P, low-Ca semi-synthetic diet. The amount absorbed in the absence of vitamin D ranged from 50 to 71% of that ingested to a range of 53 to 77% when vitamin D was given. This increase was obtained consistently with all rations and in the absence of a large need for the Mg available since most of that ingested appeared in the urine. Dietary changes, such as the addition of calcium carbonate and sodium bicarbonate, or inorganic phosphate did not affect this absorption but the addition of phytic acid reduced it slightly. Similarly, Mg

limited to the levels which could be fed without inducing catharsis was without effect on the absorption of P, or on the hydrolysis of phytic acid.

LITERATURE CITED

BOMSKOV, C., AND E. KRUGER 1931 Magnesium content of the blood serum in experimental rickets and D-hypervitaminosis. Z. Kinderheilk., *52:* 47.

DAY, H. G., AND E. V. MCCOLLUM 1939 Mineral metabolism, growth, and symptomatology of rats on a diet extremely deficient in phosphorus. J. Biol. Chem., *130:* 269.

FISKE, C. H., AND Y. SUBBA ROW 1925 The colorimetric determination of phosporus. Ibid., *66:* 375.

GASSMANN, T. 1910 Chemical investigations of healthy and rachitic bones. Z. Physiol. Chem., *70:* 161.

LEICHSENRING, J. M., L. M. NORRIS AND S. A. LAWISON 1951 Magnesium metabolism in college women: observations on the effect of calcium and phosphorus intake levels. J. Nutrition, *45:* 477.

MENDEL, L. B., AND S. R. BENEDICT 1909 The paths of excretion for inorganic compounds. IV. The excretion of magnesium. Am. J. Physiol., *25:* 1.

PRINGLE, W. J. S., AND T. MORAN 1942 Phytic acid and its destruction in baking. J. Soc. Chem. Ind., *61:* 108.

SCHMIDT, C. L. A., AND D. M. GREENBERG 1935 Occurrence, transport and regulation of calcium, magnesium, and phosphorus in the animal organism. Physiol. Rev., *15:* 297.

STEENBOCK, H., S. A. BELLIN AND W. G. WIEST 1951 Vitamin D and urinary pH. J. Biol. Chem., *193:* 843.

TIBBETTS, D. M., AND J. C. AUB 1937 Magnesium metabolism in health and disease. I. The magnesium and calcium excretion of normal individuals, also the effects of magnesium, chloride, and phosphate ions. J. Clin. Invest., *16:* 491.

VON EULER, H., AND M. RYDBOM 1931 The influence of magnesium salts on bone formation and rickets. Biochem. Z., *241:* 14.

OBSERVATIONS ON BLOOD PRESSURE AND TISSUE CHOLESTEROL FOLLOWING CHOLINE DEFICIENCY IN WEANLING RATS[1]

ARTHUR KNUDSON AND RAYMOND HARRIS

Department of Biochemistry, Albany Medical College of Union University Albany, N. Y.

(Received for publication January 7, 1955)

In 1939, Griffith and Wade demonstrated the development of hemorrhagic kidney lesions and fatty livers in weanling rats fed a choline-deficient diet. The occurrence of this syndrome was subsequently confirmed by several other investigators. Sobin and Landis ('47) determined the blood pressure of young rats maintained on diets deficient in choline for as long as 7 months and found that blood pressure remained normal. These results were confirmed by Best and Hartroft ('49) by direct measurement in weanling rats exhibiting the syndrome of acute choline deficiency and in older animals given similar diets for longer periods. When their rats were fed a diet low in choline for 5 days during the weanling period and then maintained on a normal diet for as long as 7 months, Best and Hartroft ('49) observed that more than one-third of these animals developed significant arterial hypertension. They attributed this arterial hypertension to the renal damage produced by the choline-deficient diet and stress imposed upon the damaged kidneys by the normal growth of their animals. Moses, Longabaugh and George ('50), confirming these results of Best and Hartroft, found that 71% of their rats had arterial blood pressure levels over 150 mm Hg at the time of sacrifice.

[1] Supported in part by research grant from the National Heart Institute of National Institute of Health, Public Health Service.

Our experiments were designed to study the changes in mean arterial blood pressure and tissue cholesterol of young adult rats of both sexes subjected to a choline-deficient diet during early life.

EXPERIMENTAL

In the first series of experiments, weanling rats of the Albany strain (Bryan, Klinck and Wolfe, '38) at the age of three weeks (average weight 40 gm) were placed on a low-choline diet for 5 days. This diet, somewhat similar to that used by Best and Hartroft, was prepared according to directions given by Copeland and Salmon ('46) under diet 43a and contained 3.6 mg of choline per 100 gm of diet. Pair-fed control animals received the same basal diet with the addition of 350 mg of choline per 100 gm of diet. At the end of the period on the low-choline diet, the surviving animals of the experimental group and the control animals were given a stock diet [2] for periods of three to 7 months until sacrificed. At the time of sacrifice under light nembutal anesthesia, direct blood pressure readings were obtained by cannulation of the carotid artery. Determinations of the total cholesterol and free cholesterol of the plasma, aorta, heart, kidney and liver were carried out on the experimental animals with elevated blood pressures and on a number of the control animals. Plasma cholesterol was determined by the method of Schoenheimer and Sperry ('34). The total cholesterol and free cholesterol of the tissues were determined by the Schoenheimer-Sperry method as modified by Sturgis and Knudson ('38).

A second series of experiments was then carried out, using a choline-free diet suggested by Moses, Longabaugh and George ('50). In this series, two different strains of rats were used, the so-called Albany strain and the Vanderbilt strain. These animals were sacrificed at periods of three to 7 months, and blood pressures were measured by direct cannulation of the carotid artery. Total and free cholesterol of the plasma, aorta, heart, kidney and liver were also determined on experi-

[2] Wayne dog food blocks.

mental animals with elevated blood pressures, and in a number of control animals. In the second series of experiments, both gross and microscopic examinations were made of a number of the experimental and control animals.

RESULTS

Data on blood pressures for both series of experiments are summarized in table 1. The average blood pressure of the rats

TABLE 1

Blood pressure in rats surviving 5-day period of either low-choline or choline-free diet shortly after weaning

DIET	NO. OF ANIMALS	AV. BLOOD PRESSURE AT SACRIFICE	ANIMALS WITH BLOOD PRESSURE ABOVE 124 MM Hg		
			No.	Blood pressure	Per cent of total animals
		mm Hg		*average*	
Low choline 3.6 mg of choline/ 100 gm	71	108 80–160 [1]	14	135	18
Low choline + 350 mg choline/ 100 gm (controls)	31	98 60–132	1	132	3.2
Choline-free	69	113 82–165	24	138	34.7
Choline-free + 350 mg of choline/ 100 gm (controls)	37	101 68–130	1	130	2.7

[1] Range.

in the first series of experiments on the low-choline diet was 108 mm Hg; for the rats on the control diet it was 98 mm Hg. Fourteen rats on the low-choline diet showed elevated blood pressures over 124 mm Hg.[3] The average blood pressure for this group was 135 mm Hg. Only 4 of these animals had blood

[3] Ninety-six per cent of all our control rats had blood pressures of 125 mm Hg or less, and this was used as upper limit for normal blood pressure.

pressure of 150 mm Hg or over. One registered a blood pressure of 160 mm Hg.

In the second series of experiments with the rats on a choline-free diet, the average blood pressure was 113 mm Hg; for the controls it was 101 mm Hg. Twenty-four of 69 rats in this experiment showed blood pressures above 124 mm Hg. The average blood pressure for these 24 rats was 138 mm Hg; only 4 of these had blood pressures above 150 mm Hg, and one showed a blood pressure of 165 mm Hg. There was no significant blood pressure difference in the second series between the two different strains or sexes of rats. These blood pressure results are lower than those reported by Best and Hartroft ('48) or by Moses, Longabaugh and George ('50).

Table 2 summarizes the total cholesterol and free cholesterol values in plasma, aorta, heart, kidney and liver. No significant differences were found in the total or free cholesterol values between the control rats and the rats maintained for 5 days on a low-choline or choline-free diet. However, in the rats on the choline-free diet, the total and free cholesterol in the kidney and liver were slightly higher than the controls, being on the average from 5 to 8% higher. It is doubtful whether these figures are of any particular significance.

Attention should be called to the wide range in the minimum and maximum amounts of total and free cholesterol in the aorta. Since the amount of aortic tissue, on the dry weight basis, was not more than 15 to 20 mg, duplicate determinations were impossible because of the small amount of available material. If it was less than 15 mg, the results were not used because the amount of cholesterol was below the minimum which could be determined accurately by the method used. Our results on the aorta should probably be considered as accurate only within $\pm$ 25%.

In the cholesterol determinations on the heart, kidney and liver, duplicate determinations were possible and were always within $\pm$ 5%. The range between the minimum and maximum total cholesterol and free cholesterol in these tissues was usually not more than 25%.

TABLE 2

Total cholesterol and free cholesterol in plasma and tissues of rats surviving a 5-day period of either low-choline or choline-free diet shortly after weaning and then maintained on stock diet for three to 7 months

Controls on same diets with addition of 350 mg of choline/100 gm of diet during 5-day period

DIET	NO. OF ANIMALS	PLASMA CHOLESTEROL		AORTA		HEART		KIDNEY		LIVER	
		Total	Free	Total	Free	Total	Free	Total	Free	Total	Free
		mg/ 100 ml	*mg/ 100 ml*	Total and free cholesterol as mg/100 gm dry tissue							
Low-choline	14	76	21	464	259	694	598	2251	2073	928	794
3.6 mg choline/100 gm		52–95 [1]	17–27	284–777	150–311	563–790	505–694	2092–2563	1870–2428	805–1063	704–902
Low-choline + 350 mg choline/100 gm (controls)	28	69	18	461	230	660	581	2277	2081	967	874
		56–83	15–21	282–651	197–434	550–783	520–695	2075–2485	1782–2406	830–1178	815–1051
Choline-free	24	68	19	465	265	666	611	2350	2255	1005	903
		58–78	14–24	250–724	134–427	548–718	502–654	2168–2454	2138–2387	860–1259	780–1037
Choline-free + 350 mg choline/100 gm (controls)	19	73	18	450	239	657	593	2243	2101	925	850
		55–82	14–20	345–692	154–398	543–755	503–715	2026–2517	1990–2446	817–1112	785–1050

[1] Range.

In the second series, no significant changes were observed in the microscopic examination of the internal organs except for the kidneys. Of a total of 70 experimental rats examined, severe kidney lesions were found in only 6 animals (8.6%), moderate kidney lesions in 10 animals (14.3%) and slight kidney lesions in 21 animals (30%). In the remaining 33 test rats (4.1%) and in all the control rats, no significant renal pathology occurred. The renal lesions in the experimental rats on the choline-free diet were similar to those described by Best and Hartroft ('48).

SUMMARY

In series of animals maintained on a low-choline diet for 5 days and then on a stock diet for three to 7 months, only 18% of the animals had mean arterial blood pressures above 124 mm Hg. The average for this group was 135 mm Hg and only 4 of these rats had blood pressures of 150 mm Hg or over. The highest blood pressure was 160 mm Hg.

In rats on a choline-free diet, only 34.7% of the animals showed mean arterial blood pressure above 124 mm Hg. Average mean arterial blood pressure for the group was 138 mm Hg. Four of these animals had blood pressures above 150 mm Hg, and one showed a blood pressure of 165 mm Hg.

There were no significant changes in the total cholesterol and free cholesterol in plasma, aorta, heart, kidney and liver of those animals with elevated blood pressures.

Renal lesions, varying from slight to severe, were observed in 52.9% of the rats on the choline-free diet. These lesions were similar to those described by Best and Hartroft.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. Victor Tompkins of the New York State Department of Health Laboratories, Albany, N. Y. for gross and microscopic examinations of the internal organs of a number of the experimental and control animals.

Grateful acknowledgment is made to Mr. William F. Banks, Jr. for his valuable technical assistance.

LITERATURE CITED

BEST, C. H., AND W. S. HARTROFT 1949 Nutrition, renal lesion and hypertension. Fed. Proc., *8:* 610.

BRYAN, W. R., G. H. KLINCK, JR. AND J. M. WOLFE 1938 The unusual occurrence of a high incidence of spontaneous mammary tumors in the Albany strain of rats. Am. J. Cancer, *33:* 370.

COPELAND, D. H., AND W. D. SALMON 1946 The occurrence of neoplasm in the liver, lungs and other tissues of rats as a result of prolonged choline deficiency. Am. J. Path., *22:* 1059.

GRIFFITH, W. H., AND N. J. WADE 1939 Choline metabolism. I. The occurrence and prevention of hemorrhagic degeneration in young rats on a low-choline diet. J. Biol. Chem., *131:* 567.

MOSES, C., G. M. LONGABAUGH AND R. S. GEORGE 1950 Production of hypertension following choline deficiency in weanling rats. Proc. Soc. Exp. Biol. Med., *75:* 660.

SCHOENHEIMER, R., AND W. M. SPERRY 1934 A micromethod for the determination of free and combined cholesterol. J. Biol. Chem., *106:* 745.

SOBIN, S. S., AND E. W. LANDIS 1947 Blood pressure of the rat during acute and chronic choline deficiency. Am. J. Physiol., *148:* 557.

STURGES, S., AND A. KNUDSON 1938 Application of the Schoenheimer-Sperry method to the determination of cholesterol and cholesterol esters in tissues. J. Biol. Chem., *126:* 543.

THE PRODUCTION AND STUDY OF AN ACUTE NICOTINIC ACID DEFICIENCY IN THE CALF

JOHN H. HOPPER AND B. CONNOR JOHNSON
Division of Animal Nutrition, University of Illinois, Urbana

(Received for publication January 13, 1955)

In studying various supplements to counteract a high incidence of diarrhea in calves, which they believed to be largely nutritional in origin, Phillips et al. ('41) found that the administration of high-vitamin A-potency shark liver oil, as well as nicotinic and pantothenic acids, largely eliminated the diarrhea and the resulting mortality from pneumonia. Johnson et al. ('47a) found that calves grew normally when fed a nicotinic acid-free diet containing 30% vitamin-free casein. The same authors ('47b) also reported that the urinary excretion of nicotinic acid and of an acid-hydrolyzable precursor could be increased by the administration of tryptophan and suggested nicotinic acid would be required on a lower protein ration.

Rosen et al. ('46) observed that tryptophan was a precursor of nicotinic acid synthesis in the rat. Esh and Sutton ('48), in studies with the dairy calf found that an increase in dietary tryptophan resulted in an increased urinary excretion of total nicotinic acid, indicating that tryptophan served as a precursor of niacin in the young calf as in other mammals. In the present investigation a synthetic milk diet, in which the protein was supplied by a combination of hydrolyzed and vitamin-free casein, was used to obtain and study nicotinic acid deficiency in the calf.

EXPERIMENTAL

Male calves of the various dairy breeds, which had remained with their dams 24 hours, were placed on the experimental diets (table 1). The animals were housed in individual,

TABLE 1

Composition of synthetic milk

CONSTITUENTS PER 100 GAL. OF MILK		COMPLETE VITAMIN PREMIX [1]	
$Ca(OH)_2$	908 gm	Thiamine hydrochloride	200 mg
Hydrolyzed casein [2]	7226 gm [3]	Riboflavin	400 mg
Vitamin-free casein	5260 gm [3]	Pyridoxine hydrochloride	200 mg
Methionine	196 gm	Ca pantothenate	600 mg
Gelatin	1476 gm	Nicotinic acid [4]	800 mg
		p-Aminobenzoic acid	800 mg
KOH	363 gm	Inositol	8 gm
NaOH	352 gm	Choline chloride	80 gm
		Biotin	5 mg
Cerelose	14808 gm	Pteroylglutamic acid	80 mg
		2-Methyl-1,4-naphthoquinone	80 mg
$CaCl_2 \cdot 2H_2O$	807 gm		
		Dissolved in 1000 ml of 95% ethyl alcohol, diluted to 3000 ml with distilled water and 10 ml added to each liter of "milk" at time of feeding.	
MgO	113 gm		
KH_2PO_4	340 gm		
$C_6H_8O_7 \cdot H_2O$	832 gm		
NaH_2PO_4	1851 gm		
$CuSO_4 \cdot 5H_2O$	885 mg		
KI	2509 mg		
$MnSO_4 \cdot H_2O$	4428 mg		
$ZnCl_2$	738 mg		
CaF_2	1476 mg		
Ferric citrate	103 gm		
Lard emulsion [5]	25235 gm		

[1] Vitamin B_{12} injected once a week at a level of 1 μg/kg body wt./day; 200,000 I.U. of vitamin A and 100 I.U. of vitamin E given by gelatin capsule at time animals placed on trial, and once weekly throughout trial; 50,000 I.U. vitamin D given by gelatin capsule when animals were placed on trial.

[2] Acid hydrolyzed and salt-free casein obtained from Sheffield Chemical Company, Norwich, New York.

[3] These values are computed to supply 0.17% tryptophan (Greenberg, '51). To supply 0.10% tryptophan these would be: hydrolyzed casein, 9334 gm; and vitamin-free casein, 3152 gm.

[4] Omitted from premix for some animals as indicated.

[5] A water emulsion containing 60% lard, generously supplied by Armour and Co., Chicago, Illinois through the courtesy of Mr. Byron Shinn.

wire bottom, 5- × 8-foot cages in a room in which the temperature was maintained at 76°F. The animals were fed all they would eat twice daily by nipple pails.

The synthetic milk was prepared by a modification of the procedure used by Clark ('27) and by Wiese et al. ('47). The amounts of ingredients used to prepare 100 gallons of this synthetic milk, containing 13% solids, are given in table 1. Briefly, the method of making the synthetic milk was as follows: The $Ca(OH)_2$ was added to approximately 50 gallons of water (10 to 15°C.) in a 250 gallon stainless steel container and stirred constantly. The gelatin was then dissolved in 5 gallons of boiling water and added to the tank. The hydrolyzed and vitamin-free casein and methionine were then slowly added to the solution in the tank and allowed to mix until they were in solution. The KOH and NaOH were dissolved in two and one-half gallons of water and this solution added, followed by the cerelose. $CaCl_2$ was dissolved in two and one-half gallons of water and added when the cerelose had dissolved. The remaining minerals were all dissolved in 5 gallons of water in the order listed in table 1 and then added to the tank. The lard was added as a lard in water emulsion [1] containing 60% lard. Two hundred pounds of crushed ice was added to cool the "milk" and the volume diluted to 100 gallons. The final pH should be 6.8 to 7.0. The "milk" was then drawn off into milk cans and stored in the refrigerator at 5°C. until used. Under these conditions the "milk" remained satisfactory for use for from 10 to 14 days. The composition of the synthetic milk on a dry matter basis was 24% casein, 3% gelatin, 0.4% methionine, 32.5% cerelose, 30.9% lard and 9.2% minerals.

The synthetic milk was mixed approximately once a week. The milk was warmed to 37°C. before feeding. All of the B vitamins (table 1) (except nicotinic acid, as indicated for each animal) were added to the milk at time of feeding. Vitamins A, D and E were administered as indicated in table 1. Microbiological assays (Johnson, '48) of the hydrolyzed and vita-

[1] Supplied by Armour and Co., Chicago, Ill.

min-free casein indicated both to be essentially nicotinic acid-free. Urine was collected under toluene and stored at 10°C. until assays were made.

RESULTS

Two animals, which served as positive controls, were placed on the diet supplying 0.17% of tryptophan and 2.60 mg of nicotinic acid per liter of milk. They continued to grow and eat normally for a period of 24 to 38 days while on the diet and showed no unusual symptoms.

Two of the three animals which were placed on the same diet, but deficient in nicotinic acid, started scouring very badly by the second day on the diet. They exhibited dehydration, weakness and were unable to stand by the third or 4th day.

One animal receiving the nicotinic acid-deficient diet continued to grow rather well throughout the period on trial, and no symptoms of a deficiency were noted. The appearance of symptoms and response to treatment on deficient diets can best be seen by brief individual descriptions of each animal.

Calf no. 20, a Guernsey male, received the nicotinic acid-deficient diet, which supplied 0.17% of tryptophan. The second day on trial the calf refused to eat, became very weak and was scouring badly. The third day on trial the animal was given 6 mg of nicotinic acid orally. The following day the appetite had improved and scouring had almost ceased. This treatment continued for 4 days, by which time the calf appeared to be eating normally and gaining in body weight. It was then again placed on a deficient diet, but did not exhibit any abnormal symptoms again while on trial.

Calf no. 22, a Guernsey male, received the same treatment as calf no. 20, and showed essentially the same symptoms, namely, severe scouring and loss of appetite on the second day. It showed immediate improvement on the day following nicotinic acid administration, but the deficiency symptoms could not be produced again.

Calf no. 24, a Holstein male, was placed on the deficient diet supplying 0.17% of tryptophan. This animal did not ex-

hibit any of the symptoms of the previous two animals during the 22-day period it was on trial.

As the animals could not be brought back down with a deficiency after nicotinic acid had once been given, and one calf failed to exhibit any deficiency symptoms at all, the animals were apparently synthesizing enough nicotinic acid, even from this low level of tryptophan, to prevent a recurrence of deficiency symptoms. To check this possibility, a 24-hour urine collection was made and a microbiological assay used (Johnson, '48) to determine the nicotinic acid excretion. These results are shown in table 2.

TABLE 2

Twenty-four-hour total nicotinic acid excretion of calves receiving 0.17% tryptophan

CALF NO.	NO. DAYS ON TRIAL	NO. OF DAYS SINCE NICOTINIC ACID GIVEN	24 HR. EXCRETION OF NICOTINIC ACID
			mg
20	35	21	0.68
22	28	25	1.38
24	24	24	0.87
21	30	0	3.63
25	24	0	3.87

It can be seen that the animals on the nicotinic acid-deficient diet were excreting some nicotinic acid, even though the levels were considerably lower than in the animals receiving nicotinic acid, as one would expect. The animals were apparently synthesizing enough nicotinic acid to prevent a recurrence of the symptoms.

Calves were then placed on the same diet except that the ratio of vitamin-free casein to casein hydrolysate was changed so that the diet supplied approximately 0.10% of tryptophan in the diet. The responses of the animals to this diet were as follows:

Calf no. 26, a Holstein male, was scouring very severely on the second day after being placed on the trial, appeared very weak, dehydrated, and would not eat. However, the calf did

consume considerable quantities of water when aided in drinking. No nicotinic acid was administered and the animal was dead on the morning of the third day.

Calf no. 28, a Guernsey male, received the same diet and treatment, and exhibited the same symptoms as the above animal. Five milligrams of nicotinic acid was then injected intramuscularly. Within 24 hours the animal was considerably improved and again 10 mg of nicotinic acid was injected. The animal appeared perfectly normal the following day. Administration of nicotinic acid was then stopped, and within 48 hours the animal was scouring badly. Ten milligrams of nicotinic acid was again injected, and the animal improved and was removed from the experiment in 48 hours.

Calf no. 27, a Holstein male, received the same diet plus nicotinic acid, and appeared normal and healthy for a 20-day period on the diet, gaining at the rate of 0.65 pounds per day during the last week on experiment.

DISCUSSION

The sudden appearance of deficiency symptoms in the young calf would seem to indicate that body stores of nicotinic acid are very low at time of birth. The immediate recovery following therapy is similar to that observed in the pig by Chick et al. ('38), who reported a marked improvement within 24 hours following the administration of nicotinic acid. A dehydration similar to that reported by Handler and Dann ('42) in the dog on nicotinic acid-deficient diets was also observed in the deficient calves.

No abnormal oral symptoms similar to blacktongue reported in some other species were observed at any time. All calves were autopsied, and in the post mortem examination of calf no. 26, which died from the deficiency, no disease-producing organisms were found on bacteriological examination. Examination of two of the positive control calves revealed a slight congestion of the lungs, but not enough to be serious. No symptoms, other than loss of appetite, severe scouring, dehydration, weakness and sudden death were apparent in de-

ficient calves. The rapid appearance of these symptoms, and ensuing death or marked improvement immediately following therapy, might mask or prevent the appearance of any other symptoms. One would certainly not expect to see a great change in tissue histology in such a short period.

The results seem to indicate that the young calf can meet its requirements for nicotinic acid, certainly after the first week of age, from tryptophan, when supplied at about the level of 0.2%. Of course, more studies should be conducted to definitely confirm this figure. This would be in agreement with the work of Firth and Johnson ('54) on the young pig. It would also appear that a source of nicotinic acid is very important during the first few days of the life of the calf.

SUMMARY

Using a low-tryptophan diet, we have produced and described a nicotinic acid deficiency in the young calf. Symptoms exhibited by the 4 deficient animals were a loss of appetite, severe scouring, dehydration, weakness and sudden death by the second or third day on the deficient diet.

There is a marked and rapid improvement in deficient animals following administration of nicotinic acid.

ACKNOWLEDGMENTS

The authors are grateful to Dr. J. O. Alberts and Dr. D. A. Willegan, College of Veterinary Medicine, for autopsy of the animals and to Dr. K. E. Gardner, Department of Dairy Science, for securing calves used in these trials. This investigation was supported in part by funds donated by Merck and Company, Inc., Rahway, New Jersey. Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid, choline, and vitamin B_{12} were generously supplied by Merck and Company through the courtesy of Dr. L. Michaud.

Pteroylglutamic acid was generously supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

LITERATURE CITED

CHICK, H., T. F. MACRAE, A. J. P. MARTIN AND C. J. MARTIN 1938 III. Curative action of nicotinic acid on pigs suffering from the effects of a diet consisting largely of maize. Biochem. J., *32:* 10.

CLARK, W. M. 1927 Synthetic milk as a basis of research. J. Dairy Sci., *10:* 195.

ESH, G. C., AND T. S. SUTTON 1948 The nutrition of the newborn calf. II. Effect of dietary tryptophan on the urinary excretion of niacin and its metabolites by young dairy calves. Ibid., *31:* 909.

FIRTH, J. A., AND B. C. JOHNSON 1954 Proceedings of Forty-sixth Meeting of the American Society of Animal Production.

GREENBERG, D. M. 1951 Amino acids and protein. Charles C Thomas Publishing Co., Springfield, Ill.

HANDLER, P., AND W. J. DANN 1942 The biochemical defect in nicotinic acid deficiency. J. Biol. Chem., *145:* 145.

JOHNSON, B. C. 1948 Methods of vitamin determination. Burgess Publishing Co., Minneapolis, Minn.

JOHNSON, B. C., A. C. WIESE, H. H. MITCHELL AND W. B. NEVENS 1947a The metabolism of nicotinic acid and its role in the nutrition of the calf. J. Biol. Chem., *167:* 729.

JOHNSON, B. C., H. H. MITCHELL, T. S. HAMILTON AND W. B. NEVENS 1947b Vitamin deficiencies in the calf. Fed. Proc., *6:* 410.

PHILLIPS, P. H., N. S. LUNDQUIST AND P. D. BOYER 1941 The effect of vitamin A and certain members of the B-complex upon calf scours. J. Dairy Sci., *24:* 977.

ROSEN, F., J. W. HUFF AND W. A. PERLZWEIG 1946 The effect of tryptophane on the synthesis of nicotinic acid in the rat. J. Biol. Chem., *163:* 343.

WIESE, A. C., B. C. JOHNSON, H. H. MITCHELL AND W. B. NEVENS 1947 Synthetic rations for the dairy calf. J. Dairy Sci., *30:* 87.

THE EFFECTS OF CERTAIN VEGETABLE AND ANIMAL FATS ON THE PLASMA LIPIDS OF HUMANS

J. M. R. BEVERIDGE, W. FORD CONNELL, G. A. MAYER, J. B. FIRSTBROOK AND M. S. DEWOLFE

Departments of Biochemistry and Medicine, Faculty of Medicine, Queen's University, Kingston, Ontario, Canada

ONE FIGURE

(Received for publication January 13, 1955)

The association of high plasma lipid levels and a high incidence of atherosclerosis has been noted by many investigators. Although the significance of this relationship is, in the minds of some workers debatable, nevertheless, until unequivocal proof to the contrary is presented, it seems justifiable and indeed advisable to regard high plasma lipid levels as a factor predisposing towards the development of atherosclerosis. It follows therefore that information on the control, dietary or otherwise, of these plasma components may be of great importance.

The well-known experiments of Anitschkow ('33) and of Katz and his group (Katz and Stamler, '53) in which diets high in cholesterol caused increased plasma-lipid levels and produced in rabbits and chickens lesions analogous or similar to the atherosclerotic lesions seen in humans, made this substance suspect as a factor in human food of etiologic importance in this condition. Efforts to show that dietary cholesterol plays this role have met with little success. However, another substance of the same class, neutral fat, has been implicated as an important agent influencing the level of plasma lipids in humans. The picture has been complicated by the conflicting conclusions reached by different workers on the effects of

animal fat on the one hand and of vegetable fat on the other. Thus Keys and his colleagues ('50, '52) and Hildreth et al. ('51a, b) have reported that an increase in dietary fat, whether of animal or vegetable origin, leads uniformly to higher plasma cholesterol levels. Groen and his co-workers ('52) and Kinsell et al. ('53) while they obtained similar results with diets high in animal fat have reported decreases in lipid values when similar amounts of vegetable fat were used.

In a previous experiment (Mayer, Connell, DeWolfe and Beveridge, '54) the effects of varying the level of dietary cholesterol and the level and type of fat was tested. The various dietary modifications were made by altering the levels of appropriate components of the usual food-stuffs, a regimen more or less similar to that also used by Hildreth, Mellinkoff, Blair and Hildreth ('51b). In agreement with these workers we found that an increase in the level of dietary fat in the form of vegetable fat led to an increase in plasma cholesterol levels. It was clear, however, that when dietary modifications are made in a diet comprised of the usual variety of food-stuffs, it is difficult if not impossible to attain the desirable degree of control over the proportions of fat, protein and carbohydrate that are eaten. For this reason it was decided to attempt to devise palatable homogeneous diets comprised of a relatively few components of definitely known composition and which could be accurately made up and dispensed. This procedure permitted excellent control over caloric intake and the proportions of carbohydrate, fat, and protein. The importance of the latter feature has been clearly demonstrated by the recent report of Mann et al. ('53) who showed that whereas diets with adequate levels of protein and high levels of cholesterol when fed to monkeys did not lead to any significant abnormalities, the same dietary level of cholesterol incorporated into low protein diets caused an increase in plasma lipids and led to the early development of atherosclerosis. The following report describes the relative effects of cholesterol and vegetable and animal fats when incorporated into homogeneous formula diets.

EXPERIMENTAL

The composition of the basal diet and the subsequent successive modifications are described in table 1 and in the footnote to figure 1. Whenever cholesterol was used as a supplement it was added to an appropriate amount of the fat component of the diet and dissolved by warming the fat. The proportion of calories supplied by protein was kept constant throughout by appropriate adjustment of fat and carbohydrate levels.

TABLE 1

Composition of basal diet[1] *(MD)*

Amounts required to make a 950 calorie sample

INGREDIENT	AMOUNT	PROTEIN	FAT	CARBOHYDRATE
	gm	*gm*	*gm*	*gm*
Skimmilk powder	113.0	40.0	1.2	58.8
Corn oil	15.0	...	15.0	...
Margarine	17.0	tr	13.8	tr
Dextri-maltose	72.0	...	...	71.2
		40.0	30.0	130.0
Calories	950	160.0	270.0	520.0
% of total calories		16.9	28.4	54.7

[1] A supplement of vitamins was supplied in the form of Penta Kaps (Abbott). Roughage was supplied by the addition of about 4 gm of celluflour per 950-calorie batch. Both celluflour and vitamins were mixed in with the other ingredients.

The dietary ingredients were weighed out in sufficient amounts to make up batches of the diet containing 950 calories and placed in a Waring Blendor. In order to relieve the monotony of the diet a few drops of vanilla or a teaspoonful of chocolate syrup were added as flavouring agents to a number of the samples. Sufficient water was added to make a thick slurry. The material was then homogenized, transferred to a paper carton, and preserved until required by storage at $-25°C$. The experimental diets were usually mixed with cold or hot water to make a thin milk-like fluid and the subjects regulated their consumption of this material to maintain constant body weight. The subjects were permitted ad libitum,

water, clear tea, and clear coffee. No other food was supposed to be eaten during the experimental periods. The only times when this regimen was not followed are indicated herewith. One subject had an ear infection during the first experimental period (HM) and forsook the diet for 5 meals on days two and three during which time he took only fluids and skimmed milk. He also took a sulpha drug to control his infection. Again during the second experimental period (MDC) on days two and three he went off the diet for 5 meals when he had to make a business trip. This subject again kept to a vegetarian diet except for skimmed milk. Another subject became ill on the 6th day of the 5th experimental period (HMC) and went off the diet for 5 meals. He maintained himself on fluids during this time.

Five apparently healthy, male faculty members ranging in age from 33 to 41 years, participated in the experiment. Following an initial 14-day period during which blood samples for lipid analyses were taken at 0, 4, 7, 11 and 14 days, the subjects consumed an homogenized formula diet (MD, table 1) for 11 days. During this time blood samples were taken at 0, 4, 7 and 11 days. Each individual was then allowed to eat his customary diet for a 10-day interval, blood samples being taken at 0, 4, 7, and 10 days. This was then followed by another 11-day interval on an experimental diet (MDC). This sequence was repeated throughout except towards the end of the experiment when, due to travel commitments, one or two of the rest periods were extended and some of the blood samples could not be taken during the rest periods. The last experimental diet was not ingested simultaneously by all subjects, three of them commencing 7 days later than the other two. However, the results are plotted as though consumption of the diet had been carried out simultaneously by all subjects. All blood-letting was performed between 8:00 and 8:30 A.M. prior to breakfast. Total, free and ester cholesterol were measured in the plasma by means of a modification of the Schoenheimer-Sperry procedure (Sperry and Webb, '50). Phospholipid was isolated as described by Sinclair and

Dolan ('42) and the phosphorus determined by the method of Beveridge and Johnson ('49).

RESULTS

The mean plasma-lipid values obtained during both the "rest" and experimental periods are shown in figure 1. Because of the remarkable parallelism in the behaviour of all of the lipid fractions subsequent remarks will be directed primarily to the total cholesterol component. The first experimental diet (MD), which provided 28.4% of the total calories in the form of vegetable fat, caused an average decrease in total plasma cholesterol of 33 mg per 100 ml. The addition of 200 mg of cholesterol per 950 calorie batch of diet (MDC) led to essentially the same result, an average decrease of 27 mg per 100 ml being observed. When the proportion of calories supplied by vegetable fat was increased to 58.5% (HM) the decrease in plasma cholesterol averaged 47 mg per 100 ml. In contrast to the results given above, when 58.5% of the calories were supplied by animal fat in the form of butter (HB), the plasma cholesterol values did not fall but actually increased by the 4th and 7th days and by the 11th day dropped to essentially the same concentration as observed at the beginning of the experimental period. Even a cursory inspection of the results is enough to reveal that there is undoubtedly a gross difference between the two types of fat in their effect on plasma lipid levels. A statistical analysis of the data was performed and the results confirm the statement made in the previous sentence (see footnote to fig. 1).

The possibility that the cholesterol present in the animal fat and absent from the vegetable fat might be responsible for the difference observed led us to test the effect of a diet high in vegetable fat to which an amount of cholesterol was added (HMC) equivalent to that supplied by the diet high in animal fat. However, as was the case with all the other diets containing vegetable fat there occurred a rapid decrease in plasma cholesterol, the average drop at the end of the 11th day being 38 mg per 100 ml.

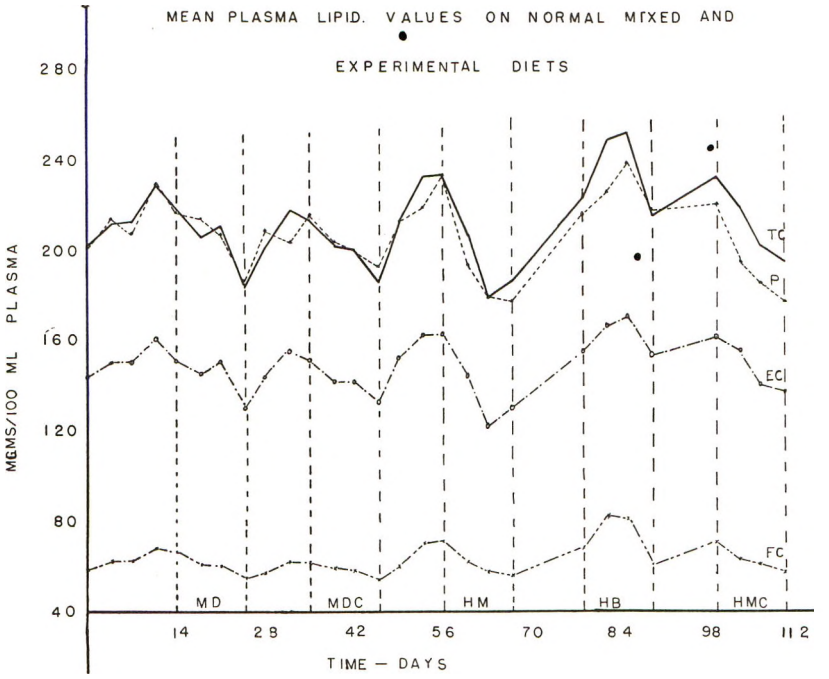


Fig. 1 Mean plasma values for total cholesterol (TC), free cholesterol (FC), ester cholesterol (EC) and phospholipid (P) of the subjects throughout the experimental and intervening or "rest" periods. The experimental periods, during which time the formula diets were consumed, are indicated by the symbols MD, MDC, HM, HB, and HMC.

The diets eaten during these periods were:

MD — basal diet in which 28.4% of the total calories was supplied in the form of vegetable fat (see table 1).

MDC — basal diet plus 200 mg cholesterol per 950-calorie batch.

HM — the percentage of calories supplied by vegetable fat was increased from 28.4 to 58.5% by substitution of corn oil for an equi-caloric amount of dextrimaltose.

HB — similar to HM except that the vegetable fat was replaced by an equi-caloric amount of butter fat.

HMC — similar to HM except that 161 mg of cholesterol was added per 950 calorie-batch, this amount of sterol being equal to that supplied in the HB ration by the butter component.

The mean differences of the individual differences between the sets of valus for plasma lipids for HB and HM, and HB and HMC, were calculated, from which the standard errors and t values were obtained (cf. Bernstein and Weatherall, '52). In both comparisons the lipid levels (TC, FC, EC, and P) for HB were significantly higher than those for HM and HMC the probability being in all cases < 0.01.

DISCUSSION

Whether or not plasma lipids will fall or rise following the transition from one diet to another depends of course upon the level of plasma lipids attained on the antecedent diet. In the present experiment, while no attempt was made to control the diet during the intermediate rest periods, each individual followed his usual dietary habits. If all factors affecting plasma lipid levels had remained constant, and of course there was no assurance of this, then it would be expected that the plasma lipids of each subject would be of approximately the same order of magnitude immediately prior to the ingestion of each experimental ration. Figure 1 shows that this assumption holds moderately well but only within certain limits, there being some variation between the initial plasma lipid levels in the various intervals. The actual average values in milligrams of cholesterol per 100 ml of plasma on day zero of each experimental period were: 217, 212, 233, 224, 232. If, therefore, precisely the same diet had been used throughout all experimental periods, other factors remaining constant, the rates of change of plasma lipid levels presumably would have been slightly different.

When vegetable fat diets with cholesterol (MDC, HMC) or without this supplement (MD, HM) were ingested the plasma lipids fell at approximately the same rates. Whether or not this drop was due to the elimination of animal fat from the diet or to a direct action of vegetable fat per se is a question that cannot be answered categorically at the present time. Further work must be done in order to clarify this problem. When the diet high in animal fat was eaten, the plasma lipids actually increased at first, thus illustrating unequivocally that there is some material in butter absent from the vegetable fat preparations that were used, that causes the plasma lipid levels to rise. After this experiment was completed Ahrens, Blankenhorn and Tsaltas ('54) reported somewhat similar findings obtained on hospital patients utilizing a dietary regimen in which some latitude was allowed with respect to the carbohydrate moiety of the ration but in which the bulk of

the protein and fat was supplied by a homogenized formula-type supplement containing milk protein [1] and either plant or animal fat.

These findings need not necessarily be looked upon as being in conflict with those of Keys et al. ('50, '52) and Hildreth et al. ('51a, b) who reported that vegetable fat and animal fat have essentially the same plasma-lipid-raising potentialities when added to a diet containing the usual variety of non-vegetarian food-stuffs. Indeed prior to the work reported here we conducted a dietary study in which various levels of cholesterol and vegetable and animal fats were incorporated into a mixed diet and, like these investigators, we found that when the dietary level of fat was increased, no matter whether it was of animal or vegetable origin, plasma lipids rose. It is suggested that although vegetable fat per se has no tendency to increase the concentration of plasma lipids, in conjunction with some unknown substance of animal origin usually present in a non-vegetarian diet it does raise the levels of these components. Since high proportions of animal fat alone when included in the simple formula diets caused an elevation of plasma lipids, it seems reasonable to assume that this unknown substance of animal origin is lipid in nature or associated with lipids. Experiments are now under way to test this hypothesis and to determine the effects of various other vegetable and animal fats on plasma lipid levels.

SUMMARY

Five apparently healthy male subjects, aged 33 to 41 years, consumed alternately mixed "free-choice" diets and homogenized "formula" diets. The latter, whose composition was accurately known, varied both in cholesterol content and in the amount and type of fat. Blood samples were taken throughout the study and the plasma analyzed for total and free cholesterol and phospholipid.

Diets containing vegetable fat, comprising either 28.4 or 58.5% of total calories, with or without supplementary chol-

[1] Lesofac (Wyeth).

esterol, led to decreases of similar magnitude in plasma lipid levels relative to the levels found on the antecedent mixed "free-choice" diets. When animal fat, in the form of butter, was given to provide 58.5% of calories, there was an actual increase in lipid levels on the 4th and 7th days, followed by a return to a level slightly below that recorded at the beginning of this dietary period.

Under the conditions of this study, there was a highly significant statistical difference between the effects of vegetable fat and animal fat on the blood lipids. A hypothesis has been presented to explain why a high dietary level of vegetable fat in a non-vegetarian diet of mixed food-stuffs causes an increase in plasma lipids whereas it has no such effect when included in a homogenized simple formula diet as the sole source of fat.

ACKNOWLEDGMENTS

Our warmest appreciation is extended to Dr. J. D. Hatcher and Dr. R. O. Hurst who although not directly concerned with work in this field cooperated loyally and conscientiously throughout the experiment.

This work was made possible by a grant obtained from the J. P. Bickell Foundation, Toronto, Ontario.

The authors gratefully acknowledge the careful technical assistance of Mrs. Ann Koen and Miss Mary White.

Grateful acknowledgment is made to the Abbott Laboratories Limited for a generous gift of Penta Kaps.

LITERATURE CITED

AHRENS, E. H., JR., D. H. BLANKENHORN AND T. T. TSALTAS 1954 Effect of human serum lipids of substituting plant for animal fat in diet. Proc. Soc. Exp. Biol. Med., *86:* 872.

ANITSCHKOW, N. 1933 Arteriosclerosis ed. E. V. Cowdry. The Macmillan Co., New York.

BERNSTEIN, L., AND M. WEATHERALL 1952 Statistics for Medical and Other Biological Students. E. & S. Livingstone Ltd., London, Great Britain.

BEVERIDGE, J. M. R., AND S. E. JOHNSON 1949 The determination of phospholipid phosphorus. Canad. J. Res., Sec. E., *27:* 159.

GROEN, J., B. K. TJIONG, C. E. KAMMINGA AND A. F. WILLEBRANDS 1952 The influence of nutrition, individuality and some other factors including various forms of stress on the serum cholesterol. An experiment of nine months duration in sixty normal human volunteers. Voeding, *13:* 556.

HILDRETH, E. A., S. M. MELLINKOFF, G. W. BLAIR AND D. M. HILDRETH 1951a Serum cholesterol in humans lowered by diets low in fat. J. Clin. Invest., *30:* 649.

———— 1951b The effect of vegetable fat ingestion on human serum cholesterol concentration. Circulation, *3:* 641.

KATZ, L. N., AND J. STAMLER 1953 Experimental Atherosclerosis. Charles C Thomas, Springfield, Illinois.

KEYS, A. 1952 Human atherosclerosis and the diet. Circulation, *5:* 115.

KEYS, A. (with the collaboration of MICKELSEN, O., E. V. O. MILLER AND C. B. CHAPMAN) 1950 The relation in man between cholesterol levels in the diet and in the blood. Science, *112:* 79.

KINSELL, L. W., G. D. MICHAELS, J. W. PARTRIDGE, L. A. BOLING, H. E. BALCH AND G. C. COCHRANE 1953 Effect upon serum cholesterol and phospholipids of diets containing large amounts of vegetable fat. J. Clin. Nutrition, *1:* 224.

MANN, G. V., S. B. ANDRUS, A. MCNALLY AND F. J. STARE 1953 Experimental atherosclerosis in cebus monkeys. J. Exp. Med., *98:* 195.

MAYER, G. A., W. F. CONNELL, M. S. DEWOLFE AND J. M. R. BEVERIDGE 1954 Diet and plasma cholesterol levels. Am. J. Clin. Nutrition, *2:* 316.

SINCLAIR, R. G., AND M. DOLAN 1942 The so called ether-insoluble phospholipids in blood and tissues. J. Biol. Chem., *142:* 659.

SPERRY, W. M., AND M. WEBB 1950 A revision of the Schoenheimer-Sperry method for cholesterol determination. Ibid., *187:* 97.