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THE INFLUENCE OF DIET UPON THE STORAGE OF VITAMIN B_{12} IN LIVER AND KIDNEY [1,2]

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(Received for publication August 1, 1958)

A recent summary indicates that vitamin B_{12} participates in a number of metabolic processes (Johnson, '58) many of which involve other vitamins and dietary constituents. Earlier reports indicated that an increase of dietary riboflavin decreased the vitamin B_{12} requirement for growth and favored an increased storage in both the liver and kidney (Cooperman et al., '52). The influence of riboflavin was attributed to the formation by bacteria in the intestine of substances with vitamin B_{12} activity (Hartman et al., '51). The requirement for vitamin B_{12} was decreased also by an adequacy of choline (Schaefer et al., '49). An interdependence of folic acid and vitamin B_{12} was found with respect to the neosynthesis and incorporation of methyl groups into nucleic acid constituents (Heinle et al., '48). These and subsequent studies dealt mainly with severe deficiencies of one or both of the vitamins.

Dietary vitamin B_{12} is stored by mammals primarily in the liver and kidney. The vitamin has been found tied to protein with globulin properties and held tenaciously in emergency states (Harte, '53; Schilling, '55). The liver content has been

[1] Supported in part by the Williams-Waterman Fund, Research Corporation, at Adelphi College and at Douglass College.

[2] This work was reported in part to the American Institute of Nutrition in Philadelphia, April, 1958. Federation Proc., *17*, part I, p. 1863.

[3] A member of the staff at Adelphi College during the early phases of this work.

successively increased by dietary increases of the vitamin (Scheid et al., '51).

It seemed desirable to use the storage of vitamin B_{12} in the liver and kidney as an index of the interrelationship between vitamin B_{12} and, separately riboflavin, folic acid and choline at levels more likely to occur in human dietaries. The storage has been measured after a period of low intake of vitamin B_{12} supplemented by either low or high amounts of the three vitamins previously mentioned.

EXPERIMENTAL PROCEDURE

The effect of riboflavin was studied first and the plan for this experiment served as a model for the two succeeding experiments in which the effects of folic acid and of choline were determined.

Male, weanling rats, of the Sherman strain, were used for the experiments. Quadruplicate litter mates, weighing 35 to 50 gm, were caged separately on raised screens and given distilled water ad libitum. For two weeks they were given the diet of Frost et al. ('52) from which the riboflavin was omitted. No iodocasein or sulfaguanidine was used at any time. They were then distributed by weight among 4 experimental diets, the total number on each diet being 10 to 12. One group was given an experimental ration, hereafter called the low-riboflavin diet, which was the diet of Frost et al. modified to contain riboflavin at the level of 0.2 mg per 100 gm of diet. In addition, 1.5 μg of vitamin B_{12} were given weekly by subcutaneous injections divided into three equal doses. A second group was given the same injection of vitamin B_{12} and the high-riboflavin diet which was identical with the low-riboflavin diet except that the riboflavin was increased to 1 mg per 100 gm of diet. A third group was continued on the riboflavin-depletion diet without vitamin B_{12} supplementation. A 4th group was given the same high-riboflavin diet but supplemented by a 100-fold increase of vitamin B_{12}, or 150 μg weekly. The combination will be called the high-riboflavin-high-vitamin B_{12} diet.

The depletion period and distribution of rats on the experimental diets for folic acid and for choline followed the same pattern with, however, the omission of a 4th group. Only 5 or 6 animals were used in a group. The low diets were modified to contain either 0.1 mg of folic acid or 0.05 gm of choline. The high diets for the three series were identical and contained 1 mg of riboflavin, 0.5 mg of folic acid and 0.1 gm of choline.

The experimental period lasted 4 weeks. The food intake was equalized between the litter mates. The animals were weighed weekly.

At the end of the experimental period blood was taken from the tail vein and the hemoglobin content was determined colorimetrically by the Newcomer ('23) method. The animals were then killed by a blow on the head. The livers and kidneys were removed immediately, blotted dry, and weighed. They were then homogenized in a minimum quantity of distilled water and lyophilized. The dry powders were weighed and stored, first in a refrigerator and later in a freezer, until assayed. Occasionally a portion of the light powder was sucked from the lyophilization flask and prevented obtaining a final dry weight. A few of the riboflavin diet samples deteriorated in storage.

The total vitamin B_{12} and "free" vitamin B_{12} were determined simultaneously on weighed aliquots of the powders resuspended in a known volume of distilled water by the method of Hutner et al. ('56) utilizing the Z strain of *Euglena gracilis*. It was found necessary to follow the method explicitly for good quadruplicate results and to heat the samples for the "free" determination at 56°C to prevent mold growth. The liver and kidney samples from each of the three experiments were assayed in random fashion in 13 assay runs. Within each run littermate samples were directly compared. The sample was determined at one level only, in quadruplicate, but was repeated in a subsequent run if the growth response was outside the limits of the standards or seemed inconsistent with values for samples from the same dietary

background. One liver sample was determined repeatedly in each of the assay runs to check on the variability of the response of the *E. gracilis* to the vitamin B_{12} standards. The average potency of this liver sample was used in conjunction with the standards to assess the potency of the other samples.

The nitrogen content of the dry powders was determined by the semi-micro Kjeldahl method. *Lactobacillus casei* was used as the test organism for assaying the riboflavin (A.O.A.C., '55) and the folic acid (Methods of Biochemical Analysis, '55) content of the livers and kidneys from the group directly concerned. The choline content was determined gravimetrically by means of *Neurospora crassa* (Hawk et al., '54).

The vitamin B_{12} concentration was calculated for liver and kidney as micrograms per milligram of dry (lyophilized) weight and per milligram of tissue N. From weights obtained during the experimental period it was possible to calculate the vitamin concentration per gram of fresh weight, and per total weight of the organ. The total vitamin B_{12} per gram of body weight was obtained by dividing the sum of the vitamin B_{12} values for the total liver and the total kidney by the grams of body weight. The small amount of this vitamin in the other tissues and in the blood would not be included in this value.

RESULTS AND DISCUSSION

The results obtained from the three studies are given in tables 1, 2 and 3.

Effect of riboflavin. The 5-fold dietary increase of riboflavin for a 4-week period resulted in a small increase of total vitamin B_{12} in both liver and kidney when calculated per gram of dry tissue or nitrogen. Statistically, the differences were not significant, the "t" value (the difference of the means divided by the standard error of the difference) being 1.2. However, an increase of both total and free vitamin B_{12}, determined independently, in both liver and kidney seemed to indicate a sparing action of riboflavin at sub-optimal levels of vitamin B_{12}. The amount of vitamin B_{12} per gram of fresh tissue or per total organ increased for the liver but decreased

TABLE 1

Effect of varying riboflavin upon the vitamin B_{12} content of liver and kidney

	NO. OF RATS	LOW RIBOFLAVIN [1]	NO. OF RATS	HIGH RIBOFLAVIN [1]	NO. OF RATS	RIBOFLAVIN DEFICIENT [1]	NO. OF RATS	HIGH RIBOFLAVIN-HIGH-VITAMIN B_{12} [1]
Liver								
Total vitamin B_{12}, μg/gm $\times$ 10^{-2} dry tissue	11	46.7 $\pm$ 7.8 [2]	10	54.7 $\pm$ 6.4	10	29.7 $\pm$ 5.0	5	93.9 $\pm$ 11.5
Free vitamin B_{12}, μg/gm $\times$ 10^{-2} dry tissue	8	41.4 $\pm$ 8.6	8	55.1 $\pm$ 7.2	6	18.4 $\pm$ 3.4	4	60.4 $\pm$ 1.5
Total vitamin B_{12}, μg/gm $\times$ 10^{-2} fresh tissue	9	10.8 $\pm$ 2.8	8	13.6 $\pm$ 1.6	8	5.0 $\pm$ 1.0	3	22.1 $\pm$ 2.0
Total vitamin B_{12}, μg/gm N	11	5.22 $\pm$ 0.92	10	5.31 $\pm$ 0.74	10	3.27 $\pm$ 0.60	5	8.67 $\pm$ 2.07
Riboflavin, μg/gm dry tissue	6	74 $\pm$ 3	5	70 $\pm$ 4	7	68 $\pm$ 9	5	76 $\pm$ 3
Kidney								
Total vitamin B_{12}, μg/gm $\times$ 10^{-2} dry tissue	9	288 $\pm$ 61	10	320 $\pm$ 62	8	62 $\pm$ 7	5	565 $\pm$ 108
Free vitamin B_{12}, μg/gm $\times$ 10^{-2} dry tissue	9	227 $\pm$ 47	10	270 $\pm$ 69	7	54 $\pm$ 14	5	374 $\pm$ 114
Total vitamin B_{12}, μg/gm $\times$ 10^{-2} fresh tissue	7	76.6 $\pm$ 13.7	8	63.3 $\pm$ 15.8	5	13.2 $\pm$ 3.2	3	101.6 $\pm$ 5.9
Total vitamin B_{12}, μg/gm N	9	29.16 $\pm$ 6.52	10	31.55 $\pm$ 5.04	8	6.29 $\pm$ 0.77	5	63.64 $\pm$ 12.58
Riboflavin, μg/gm dry tissue	4	124 $\pm$ 11	5	103 $\pm$ 7	6	79 $\pm$ 10	5	84 $\pm$ 7
Animal								
Total vitamin B_{12}, μg/gm $\times$ 10^{-2} body wt	6	98.0 $\pm$ 26.9	6	103.0 $\pm$ 25.4	5	26.6 $\pm$ 5.7	3	155.0 $\pm$ 20.8
Hemoglobin, gm/100 ml	3	16.7 $\pm$ 0.3	7	17.3 $\pm$ 0.4	5	17.4 $\pm$ 0.1	3	18.2 $\pm$ 0.1
Gain in weight, 4 weeks	11	104 $\pm$ 8	10	114 $\pm$ 8	10	73 $\pm$ 10	5	132 $\pm$ 11

[1] The low-riboflavin diet supplied 0.2 mg of riboflavin/100 gm of diet and 1.5 μg of vitamin B_{12}/week; the high-riboflavin diet supplied 1.0 mg and 1.5 μg and the high-riboflavin-high-vitamin B_{12} diet 1.0 mg and 150 μg of each vitamin respectively.

[2] Mean $\pm$ standard error.

for the kidney due to a higher moisture content and lower total kidney weight.

The increment by which the free vitamin B_{12} increased on the high-riboflavin diet was larger than that for the total vitamin B_{12}, the percentage of free vitamin B_{12} rising from 88 to 100% in the liver and from 79 to 81% in the kidney. Additional studies would be required to determine whether the change made the vitamin more or less available for metabolism.

The nature of the free vitamin B_{12} is not clear from these studies. The vitamin B_{12} of human liver has been found to be completely available to the *E. gracilis* (Pitney et al., '55). Concurrent experiments, using ionophoresis and paper chromatography, have indicated the presence of several components in the liver and kidney powders. Additional studies are being conducted with the aim of finding some pattern consistent with the dietary changes.

The content of both total and free vitamin B_{12} in both liver and kidney for the deficient diet was significantly lower than that for either the high or low diet, "t" being two for the liver and over three for the kidneys. The percentage of free vitamin B_{12} dropped to 62 in the liver. The diet of Frost et al. ('52) seemed to provide the proper basis for the study. Coprophagy (Barnes and Fiala, '58) would seem not to have been an interfering factor in these studies.

The high-riboflavin diet supplemented with 1.5 μg of vitamin B_{12} weekly did not produce maximum storage. Markedly higher levels of the vitamin, both free and bound, were found when the vitamin B_{12} was increased 10-fold by the high-riboflavin-high-vitamin B_{12} diet.

The increase of stored vitamin B_{12} on the high-riboflavin diet could have resulted from an increased release of vitamin B_{12} active fragments from riboflavin during digestion. However, an actual decrease of stored riboflavin on this diet indicates a metabolic interrelationship.

Effect of folic acid. In the second series, a 5-fold increase of folic acid showed a small, statistically non-significant, in-

TABLE 2

Effect of varying folic acid upon the vitamin B_{12} content of liver and kidney

	NO. OF RATS	LOW FOLIC ACID [1]	NO. OF RATS	HIGH FOLIC ACID [1]	NO. OF RATS	FOLIC ACID DEFICIENT [1]
Liver						
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ dry tissue	6	54.9 $\pm$ 6.8 [2]	6	57.0 $\pm$ 7.9	6	30.0 $\pm$ 8.0
Free vitamin B_{12}, μg/gm $\times 10^{-2}$ dry tissue	6	43.8 $\pm$ 6.0	6	43.5 $\pm$ 8.4	6	20.4 $\pm$ 8.3
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ fresh tissue	6	16.1 $\pm$ 1.9	5	17.5 $\pm$ 2.7	4	6.4 $\pm$ 1.4
Total vitamin B_{12}, μg/gm N	6	5.64 $\pm$ 0.70	6	5.90 $\pm$ 0.70	5	3.48 $\pm$ 1.07
Folic acid, μg/gm $\times 10^{-3}$ dry tissue	5	7.3 $\pm$ 1.2	5	7.7 $\pm$ 1.8	5	3.9 $\pm$ 0.6
Kidney						
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ dry tissue	5	242 $\pm$ 83	4	315 $\pm$ 88	4	39 $\pm$ 7
Free vitamin B_{12}, μg/gm $\times 10^{-2}$ dry tissue	4	260 $\pm$ 42	4	304 $\pm$ 94	4	26 $\pm$ 10
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ fresh tissue	5	57.5 $\pm$ 20.4	4	53.0 $\pm$ 20.1	3	9.4 $\pm$ 1.0
Total vitamin B_{12}, μg/gm N	5	21.94 $\pm$ 7.63	4	27.12 $\pm$ 1.72	4	3.74 $\pm$ 0.64
Folic acid, μg/gm $\times 10^{-3}$ dry tissue	4	4.5 $\pm$ 0.5	5	7.1 $\pm$ 1.3	4	5.3 $\pm$ 1.1
Animal						
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ body wt.	5	106.5 $\pm$ 18.3	4	108.6 $\pm$ 23.2	2	43.8 $\pm$ 14.0
Hemoglobin, gm/100 ml	6	18.1 $\pm$ 0.5	6	18.6 $\pm$ 0.7	6	17.7 $\pm$ 0.4
Gain in weight, 4 weeks	6	114 $\pm$ 8	6	120 $\pm$ 3	6	114 $\pm$ 7

[1] The low-folic acid diet supplied 0.1 mg folic acid/100 gm and 1.5 μg vitamin B_{12}/week; the high-folic acid diet supplied 0.5 mg and 1.5 μg of each vitamin respectively.

[2] Mean $\pm$ standard error.

crease of total vitamin B_{12} in the liver and kidney. The increase occurred primarily with the bound form since the percentage of free vitamin B_{12} dropped. A decrease of total vitamin B_{12} in the kidney, calculated on either a fresh weight or total organ basis was similar to that found for the high-riboflavin diet. These results indicate that a vitamin B_{12} content based on either dry weight or nitrogen content gives the better indication of the dietary effects.

The increase of stored vitamin B_{12} on the high-folic acid diet was of the same magnitude as that on the high-riboflavin diet but was attended by a different metabolic pattern. Dietary riboflavin favored the increase of free vitamin B_{12} while folic acid favored the bound form. Only with increased folic acid were there found steady, but small, increases of hemoglobin and of stored folic acid. Folic acid seemed to have a slight sparing action upon vitamin B_{12} at suboptimal levels which was related to the maintenance of hemoglobin.

Effect of choline. In the third series, the two-fold increase of choline caused a significant decrease of total and free vitamin B_{12} in both liver and kidney to a level approximately that found for the deficient diet. The decreased storage occurred with little or no change in the hemoglobin level or in the storage of choline in the liver and kidney. The percentage of nitrogen in the dry tissue dropped but only by 0.2% in the liver and 0.4% in the kidney.

The decreased storage of vitamin B_{12} may have resulted from renal lesions and fatty infiltration of the organs during the two-weeks depletion period (Moore, '57) on a choline-free diet. The slight decrease in nitrogen content would contraindicate such changes being a major factor. Moreover, the animals on the low-choline diet were depleted in a similar manner and yet were able to deposit vitamin B_{12} at a level comparable to that found in animals on either the low-riboflavin or low-folic acid diets. The level of 0.1 gm of choline per 100 gm of diet was not harmful *per se* since the low and high diets in the first two series contained that level of choline. The inbalance of high choline and low vitamin B_{12} following

TABLE 3

Effect of varying choline upon the vitamin B_{12} content of liver and kidney

	NO. OF RATS	LOW CHOLINE [1]	NO. OF RATS	HIGH CHOLINE [1]	NO. OF RATS	CHOLINE DEFICIENT [1]
Liver						
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ dry tissue	5	35.4 $\pm$ 3.8 [2]	7	30.1 $\pm$ 3.2	5	12.7 $\pm$ 1.3
Free vitamin B_{12}, μg/gm $\times 10^{-2}$ dry tissue	6	32.0 $\pm$ 4.9	6	23.2 $\pm$ 4.0	5	11.5 $\pm$ 1.1
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ fresh tissue	5	9.5 $\pm$ 1.3	7	8.2 $\pm$ 1.0	4	3.7 $\pm$ 0.4
Total vitamin B_{12}, μg/gm N	5	3.81 $\pm$ 0.48	7	3.35 $\pm$ 0.36	6	1.69 $\pm$ 0.13
Choline, mg/gm dry tissue	4	0.61 $\pm$ 0.04	4	0.64 $\pm$ 0.07	4	0.51 $\pm$ 0.05
Kidney						
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ dry tissue	6	328 $\pm$ 91	7	90 $\pm$ 23	5	81 $\pm$ 15
Free vitamin B_{12}, μg/gm $\times 10^{-2}$ dry tissue	6	322 $\pm$ 77	5	92 $\pm$ 38	5	66 $\pm$ 16
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ fresh tissue	6	82 $\pm$ 27	6	21 $\pm$ 4	5	12 $\pm$ 4
Total vitamin B_{12}, μg/gm N	6	30.46 $\pm$ 2.99	6	9.32 $\pm$ 2.21	5	7.79 $\pm$ 1.48
Choline, mg/gm dry tissue	3	0.74 $\pm$ 0.08	5	0.67 $\pm$ 0.05	4	0.55 $\pm$ 0.03
Animal						
Total vitamin B_{12}, μg/gm $\times 10^{-2}$ body wt.	6	106.3 $\pm$ 24.1	6	51.2 $\pm$ 34	5	37.6 $\pm$ 5.9
Hemoglobin, gm/100 ml	6	17.3 $\pm$ 2.6	7	17.3 $\pm$ 0.5	6	17.8 $\pm$ 0.6
Gain in weight, 4 weeks	6	104 $\pm$ 3	7	109 $\pm$ 2	6	118 $\pm$ 7

[1] The low-choline diet supplied 0.05 gm choline/100 gm diet and 1.5 μg vitamin B_{12}/week; the high-choline diet supplied 0.10 gm and 1.5 μg of each vitamin respectively.

[2] Mean $\pm$ standard error.

a depletion period may have prevented the normal storage of vitamin B_{12}, or provided an abnormal demand for methyl group mobilization, or induced increased excretion. The present studies provide for no interpretation. With the experimental method employed, choline exerted an influence unfavorable to the storage of vitamin B_{12} in liver and kidney.

A comparison of equivalent results from each of the three series provides some interesting data. For the deficient and the low diets the highest values for both the total and free vitamin B_{12} were found in the livers from the folic acid diets and in the kidneys from the choline diets. The content of nitrogen, on a dry weight basis, was highest for the organs from the folic acid diets and lowest for the riboflavin diets. For each of the three dietary levels, the total vitamin B_{12} calculated per gram of body weight was highest for the folic acid diets.

The lowest value of vitamin B_{12} on a fresh weight basis was 0.037 μg per gm of fresh liver for the diet deficient in vitamin B_{12} and choline. This value is in excess of the values 0.026 μg (Scheid et al., '51) and 0.005 μg (Burns and Salmon, '56) reported for this organ from vitamin B_{12}-deficient diets. The inclusion of iodocasein and sulfaguanidine in the diet of Frost et al. ('52) probably would have produced lower vitamin values but could have interfered with measuring any small changes resulting from interrelated processes. The highest value of vitamin B_{12} was 0.175 μg per gm of liver on the high-folic acid diet and was almost three-fold the value reported for rats on a diet containing 24 μg of vitamin B_{12} per kilogram (Scheid et al., '51). The values for the livers from all of the low and high diets fell within the range of 0.11 to 0.47 μg per gm reported for fresh human liver (Pitney et al., '55).

SUMMARY

Litter mate weanling rats were depleted for two weeks of vitamin B_{12} and either riboflavin, folic acid or choline. For a 4-week experimental period they were injected with 1.5 μg of vitamin B_{12} weekly and fed either a diet low in one of the

vitamins being studied (0.2 mg riboflavin, or 0.1 mg folic acid or 0.05 gm choline per 100 gm of diet) or the same diet with a high level of the vitamin (1.0 mg riboflavin, 0.5 mg folic acid, or 0.1 gm choline per 100 gm). The vitamin B_{12} content of the lyophilized livers and kidneys was determined, using the Z strain of *E. gracilis* with respect to both total and free vitamin. Littermates were continued for the additional 4-week period on the deficient diet. For all three vitamins the deficient rats showed a significantly lower level of both total and free vitamin B_{12}. The dietary increase of riboflavin or of folic acid from low to higher levels resulted in small increases of total and free vitamin B_{12} in both liver and kidney on a dry weight and on a nitrogen basis. There was evidence of interrelationship. On the high-riboflavin diet the free vitamin B_{12} increased to 100% in the liver and remained at 80% in the kidney. On the high-folic acid diet the free vitamin B_{12} remained close to 80% in the liver and 100% in the kidney. While the vitamin B_{12} content of the low-choline diet approximated those for the two other low diets, the high diet showed a significant decrease which could not be accounted for by possible lesions produced during the depletion period. The free vitamin B_{12} dropped from 91 to 77% in the liver and remained at 100% in the kidney.

The storage of vitamin B_{12} in liver and kidney was influenced not only by the vitamin B_{12} content of the diet but by constituents such as riboflavin, folic acid and choline at levels below optimal but sufficiently high to prevent deficiency symptoms.

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ENZYMES IN PROTEIN DEPLETION

III. ENZYMES OF BRAIN, KIDNEY, SKELETAL MUSCLE AND SPLEEN [1,2]

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In the first paper of this series (Wainio, Eichel, Eichel, Person, Estes and Allison, '53) it was shown that the unit activities (per milligram of nitrogen) and the total activities (per organ) of several oxidative enzymes of liver were decreased as a result of protein depletion. Cytochrome oxidase was an exception. In the second paper (Wainio, Allison, Eichel, Person and Rowley, '54) it was shown that the unit activities of three oxidative enzymes of rat heart ventricle were unaffected by protein depletion. The total activities, however, were proportional to the total protein, i.e., the depleted animals had smaller hearts which contained less total protein and less enzyme activity.

There has been only one study in protein depletion which has involved the brain, kidney, skeletal muscle or spleen. Bargoni, Cafiero, DiBella, DeMori and Grillo ('52) studied the phosphomonoesterase, pyrophosphatase, adenosinetri-

[1] This work was supported in part by a grant from the Nutrition Foundation.

[2] In the first paper of this series (Wainio, Eichel, Eichel, Person, Estes and Allison, '53) the total activities should all be divided by 6.25. In the second paper (Wainio, Allison, Eichel, Person and Rowley, '54) the DPNH-cytochrome c reductase values should be multiplied by a factor of 10^{-9}.

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phosphatase, dipeptidase, succinic dehydrogenase, and catalase activities of rat brain, lung and kidney, the esterase activities of lung and kidney, and the choline oxidase activity of kidney. They found that the unit activities of the enzymes of brain and kidney (ATPase excepted) were unchanged in protein depletion.

The present investigation is concerned with cytochrome oxidase, succinate-cytochrome c reductase and DPNH-cytochrome c reductase in brain, kidney, skeletal muscle and spleen, cholinesterase in brain, D-amino acid oxidase in kidney and aldolase in skeletal muscle and heart ventricle.

EXPERIMENTAL

Male albino rats were obtained from the Wistar Institute colony. They weighed approximately 250 gm when received, and they were treated in the manner previously described (Wainio et al., '53). In each experiment 5 rats were depleted by feeding them the protein-free diet; 5 rats were pair-fed the complete diet against the depleted animals; and 5 rats were fed the complete diet ad libitum.

The solids of the complete diet were 18% casein, 13.4% sucrose, 20.2% glucose, 18.2% dextrin, 24.2% lard, 1.7% salt mix (Wesson, '32), 1.0% cod liver oil, 3.3% agar, and the known vitamins. The agar was dissolved in an amount of water equal to 1.4 times the weight of the dry constituents. There was an isocaloric substitution of glucose for protein in the protein-free diet.

A daily record of food consumption and a weekly record of body weights were kept.

At the end of 7 weeks, one rat out of each group was sacrificed by decapitation on each of 5 successive days. The brain, when used, was scooped out with the rounded end of a scalpel to the foramen magnum. Both kidneys were removed without their capsules. The gastrocnemius was used as a representative of skeletal muscle, having been cut from its tendinous attachment at the level of the ankle joint. No attempt was made to remove the extremely thin splenic capsule. The heart

ventricles, although free of the auricles, included the atrioventricular septa and the associated valves. The organs were weighed and a weighed portion was homogenized in a Ten Broeck glass homogenizer with the appropriate diluant according to the method of assay.

Total nitrogen was determined by the microKjeldahl method. The factor 6.25 was used to convert nitrogen to protein.

Cholinesterase was determined by the method of Alles and Hawes ('40), cytochrome oxidase by the method presented in the second paper (Wainio et al., '54), and succinate-cytochrome c reductase, DPNH-cytochrome c reductase and D-amino acid oxidase by the methods presented in the first paper of this series (Wainio et al., '53). Aldolase was determined by the method of Sibley and Lehninger ('49).

RESULTS

The average total food intakes of these animals and their average final body weights were very much like those of the animals discussed in the second paper of this series (Wainio et al., '54).

The average organ weights and their average total proteins are presented in table 1. With the possible exception of brain, the organs of the depleted animals were significantly smaller in weight than those of their pair-fed or ad libitum controls. There seemed to be very little difference between the organ weights of the pair-fed and ad libitum animals. However, with brain again excepted, the former might have had slightly smaller organs, and this was probably a reflection of the smaller final average body weights of the pair-fed animals.

The average total protein contents of the organs paralleled very closely the average total weights, probably indicating minor changes in water content. For example, a comparison of the brains of the depleted and pair-fed animals revealed that the brains of the former weighed less by 9% and contained 10% less protein, while the spleens of the former weighed less by 37% and contained 41% less protein.

The results of the enzyme assays are presented in table 2. Although it was felt that 5 animals in a group was too small a number to give a reliable "p" value, the following general conclusions seem warranted because the changes were observed in almost all instances with every enzyme assayed.

Food restriction (pair-fed vs. ad libitum-fed animals) had little effect on the unit activities of the enzymes: the unit activities were sometimes larger and sometimes smaller. It

TABLE 1

The effect of protein depletion on the weight and total protein of some tissues of the rat

TISSUE	GROUP	RAT NOS.	AVERAGE TOTAL WEIGHT	AVERAGE TOTAL PROTEIN
			gm	*gm*
Brain	Depleted	81–85	1.64 ± 0.013 [1]	0.199 ± 0.003 [1]
	Pair-fed	86–90	1.77 ± 0.003	0.207 ± 0.003
	Ad libitum	91–95	1.73 ± 0.012	0.200 ± 0.003
Brain	Depleted	96–100	1.65 ± 0.036	0.205 ± 0.013
	Pair-fed	101–105	1.81 ± 0.026	0.228 ± 0.005
	Ad libitum	106–110	1.78 ± 0.049	0.218 ± 0.002
Kidney	Depleted	31–35	1.62 ± 0.158	0.21 ± 0.008
	Pair-fed	36–40	2.52 ± 0.178	0.33 ± 0.007
	Ad libitum	41–45	2.98 ± 0.146	0.37 ± 0.002
Kidney	Depleted	46–50	1.28 ± 0.010	0.20 ± 0.010
	Pair-fed	51–55	2.25 ± 0.133	0.33 ± 0.013
	Ad libitum	56–60	2.41 ± 0.072	0.38 ± 0.012
Kidney	Depleted	96–100	1.109 ± 0.055	0.210 ± 0.011
	Pair-fed	101–105	1.772 ± 0.054	0.340 ± 0.013
	Ad libitum	106–110	1.977 ± 0.093	0.393 ± 0.024
Skeletal muscle	Depleted	96–100	1.035 ± 0.054	0.228 ± 0.018
	Pair-fed	101–105	1.796 ± 0.022	0.394 ± 0.008
	Ad libitum	106–110	1.967 ± 0.074	0.404 ± 0.025
Skeletal muscle	Depleted	111–115	1.147 ± 0.054	0.227 ± 0.020
	Pair-fed	116–120	1.796 ± 0.155	0.394 ± 0.032
	Ad libitum	121–125	1.832 ± 0.091	0.404 ± 0.016
Spleen	Depleted	111–115	0.290 ± 0.021	0.051 ± 0.004
	Pair-fed	116–120	0.460 ± 0.034	0.086 ± 0.004
	Ad libitum	121–125	0.545 ± 0.006	0.098 ± 0.000
Heart ventricle	Depleted	96–100	0.566 ± 0.036	0.113 ± 0.017
	Pair-fed	101–105	0.778 ± 0.017	0.153 ± 0.003
	Ad libitum	106–110	0.885 ± 0.052	0.187 ± 0.013

[1] Mean and standard error of the mean.

did, however, slightly lower the total enzyme activities, probably because the organs of the pair-fed animals were somewhat smaller than the organs of the animals fed ad libitum.

The enzymes of the brain were the most resistant to the stress of protein depletion (cf. depleted and pair-fed animals). The unit activities of the 4 enzymes studied here were unaffected. Even the total activities, which are the product of the unit activities and the size of the organ, were reduced on the average by only 5 to 10%. The enzymes of the kidney, the skeletal muscle and the spleen were all affected about equally. The unit activities were reduced by about 10 to 20% and, since these organs of the depleted animals were smaller than those of their controls, the total enzyme activities were reduced by about 50 to 60%.

It is to be noted by comparison with the results obtained in the earlier papers that the enzymes of the liver, cytochrome oxidase excepted, are very labile and that heart ventricle occupies a position intermediate between brain on the one hand and kidney, skeletal muscle and spleen on the other. The unit enzyme activities of the heart ventricles are fairly well maintained, while the total activities are reduced due to the smaller size of the organs in the depleted animals. This is in keeping with the concept that the most vital organs are preferentially maintained.

SUMMARY

The cytochrome oxidase, succinate-cytochrome c reductase, and DPNH-cytochrome c reductase activities of brain, kidney, skeletal muscle and spleen, and cholinesterase of brain, D-amino acid oxidase of kidney, and aldolase of skeletal muscle and heart ventricle were assayed after rats had been fed a protein-free diet for 49 days. The effects of sub-acute food restriction were interpreted with the aid of pair-fed animals which were given a diet containing 18% casein. Animals fed the 18% casein diet ad libitum served as further controls.

Food restriction alone had little or no effect on the unit activities of the enzymes. Some unit activities rose slightly, others fell. Food restriction did, however, slightly decrease

TABLE 2

The effect of protein depletion on the enzyme activities of some tissues of the rat

TISSUE	ENZYME	GROUP	RAT NOS.	UNIT ACTIVITY [1]	TOTAL ACTIVITY [2]
Brain	Cholinesterase [3]	Depleted	81–85	0.401 ± 0.011 [4]	12.71 ± 0.340 [4]
		Pair-fed	86–90	0.418 ± 0.009	13.76 ± 0.294
		Ad libitum	91–95	0.456 ± 0.010	14.62 ± 0.430
Brain	Cytochrome oxidase [5]	Depleted	96–100	0.0618 ± 0.0053	2.02 ± 0.214
		Pair-fed	101–105	0.0591 ± 0.0024	2.18 ± 0.192
		Ad libitum	106–110	0.0679 ± 0.0047	2.37 ± 0.126
Brain	Succinate cytochrome c reductase [5]	Depleted	96–100	0.0882 ± 0.0126	2.87 ± 0.396
		Pair-fed	101–105	0.0837 ± 0.0085	3.03 ± 0.319
		Ad libitum	106–110	0.1105 ± 0.0082	3.82 ± 0.320
Brain	DPNH-cytochrome c reductase [5]	Depleted	97–100	0.0385 ± 0.0061	1.253 ± 0.184
		Pair-fed	101–105	0.0373 ± 0.0039	1.360 ± 0.142
		Ad libitum	106–110	0.0322 ± 0.0052	1.106 ± 0.142
Kidney	D-amino acid oxidase [6]	Depleted	31–35	26.80 ± 0.91	5,468 ± 237
		Pair-fed	36–40	29.33 ± 1.15	9,746 ± 277
		Ad libitum	41–45	28.99 ± 1.09	10,610 ± 348
Kidney	D-amino acid oxidase [6]	Depleted	46–50	25.50 ± 1.40	5,031 ± 231
		Pair-fed	51–55	29.40 ± 1.21	9,649 ± 718
		Ad libitum	57–60	30.24 ± 1.42	11,684 ± 725
Kidney	Cytochrome oxidase [5]	Depleted	96–100	1.51 ± 0.16	52.0 ± 8.0
		Pair-fed	101–105	2.12 ± 0.12	114.6 ± 4.2
		Ad libitum	106–110	1.95 ± 0.21	122.9 ± 15.2
Kidney	Succinate cytochrome c reductase [5]	Depleted	96–100	0.176 ± 0.014	5.99 ± 0.62
		Pair-fed	101–105	0.215 ± 0.008	12.41 ± 1.01
		Ad libitum	106–110	0.224 ± 0.024	13.79 ± 0.55
Kidney	DPNH-cytochrome c reductase [5]	Depleted	96–100	0.0825 ± 0.0066	2.78 ± 0.089
		Pair-fed	101–105	0.0879 ± 0.0044	4.78 ± 0.061
		Ad libitum	106–110	0.1033 ± 0.0070	6.55 ± 0.075

Skeletal muscle	Aldolase [7]	Depleted	96–100	40,700 ± 1,800	42,300 ± 4,100
		Pair-fed	101–105	53,000 ± 6,300	94,900 ± 10,300
		Ad libitum	106–110	53,800 ± 5,200	106,700 ± 12,800
Skeletal muscle	Cytochrome oxidase [5]	Depleted	111–115	0.661 ± 0.112	24.29 ± 4.74
		Pair-fed	116–120	0.774 ± 0.086	48.75 ± 9.08
		Ad libitum	121–125	0.718 ± 0.083	47.03 ± 6.55
Skeletal muscle	Succinate cytochrome c reductase [5]	Depleted	111–115	0.133 ± 0.026	4.84 ± 1.32
		Pair-fed	116–120	0.168 ± 0.018	10.58 ± 1.56
		Ad libitum	121–125	0.134 ± 0.016	8.58 ± 0.082
Skeletal muscle	DPNH-cytochrome c reductase [5]	Depleted	111–115	0.0227 ± 0.0009	0.826 ± 0.079
		Pair-fed	116–120	0.0193 ± 0.0012	1.239 ± 0.177
		Ad libitum	121–125	0.0163 ± 0.0028	1.026 ± 0.134
Spleen	Cytochrome oxidase [5]	Depleted	111–115	0.575 ± 0.052	4.81 ± 0.94
		Pair-fed	116–120	0.564 ± 0.056	7.79 ± 1.08
		Ad libitum	121–125	0.555 ± 0.017	8.69 ± 0.37
Spleen	Succinate cytochrome c reductase [5]	Depleted	111–115	0.069 ± 0.0036	0.563 ± 0.048
		Pair-fed	116–120	0.085 ± 0.0034	1.168 ± 0.116
		Ad libitum	121–125	0.082 ± 0.0059	1.285 ± 0.099
Spleen	DPNH-cytochrome c reductase [5]	Depleted	111–115	0.0443 ± 0.0027	0.357 ± 0.016
		Pair-fed	116–120	0.0512 ± 0.0038	0.692 ± 0.061
		Ad libitum	121–125	0.0512 ± 0.0015	0.801 ± 0.024
Heart ventricle	Aldolase [7]	Depleted	96–100	7,880 ± 310	4,490 ± 420
		Pair-fed	101–105	8,270 ± 580	6,470 ± 540
		Ad libitum	106–110	9,140 ± 470	8,160 ± 840

[1] Per milligram nitrogen, except for aldolase which is expressed per gram wet wt.

[2] Per organ.

[3] Expressed as milliliters of 0.0189 N NaOH per 30 min.

[4] Mean and standard error of the mean.

[5] First order velocity constant (sec.$^{-1}$). The unit activities are based on the nitrogen in 1 ml of reaction mixture.

[6] Expressed as μl O_2 per hr.

[7] Expressed as mm [3] hexose diphosphate split per hr.

the total enzyme activities, since the organs of the pair-fed animals (brain excepted) were somewhat smaller than the organs of the animals fed ad libitum.

Protein depletion with its attendant food restriction had no effect on the unit activities of the enzymes of brain, but it did lower the activities of the enzymes of kidney, skeletal muscle and spleen by about 10 to 20%. Since the weights of the organs were also reduced by protein depletion, brain excepted, the total enzyme activities of kidney, skeletal muscle and spleen were reduced by about 50 to 60%. The total enzyme activities of brain were reduced by about 5 to 10%.

A comparison of these results with those presented in the first two papers of this series reveals that the total protein and enzymes of liver are the most labile in protein depletion (cytochrome oxidase excepted), while the total protein and cholinesterase of brain are very resistant. The total protein and enzymes of heart ventricle are more resistant than those of kidney, skeletal muscle and spleen, and almost as resistant as those of brain.

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SERUM GLUTAMIC-OXALACETIC TRANSAMINASE ACTIVITY OF VITAMIN B_6-DEFICIENT RATS [1, 2]

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Recent observations on the importance of vitamin B_6 in pregnancy, infant nutrition, atherosclerosis, antibody formation and isonicotinyl hydrazide and radiation therapy have focused attention on the need for methods to measure the state of vitamin B_6 nutrition. A deficiency of vitamin B_6 is difficult to detect because the clinical signs are similar to those caused by deficiencies of other nutrients (Vilter et al., '53). Of the biochemical tests available for vitamin B_6 deficiency, probably the most satisfactory one is based on the excretion of xanthurenic acid after administering a test dose of tryptophan (Vilter, '55). This test has disadvantages in that it is affected by niacin and protein intakes (Heimberg et al., '50), the tryptophan dose frequently causes nausea or sleepiness (Wachstein and Gudaitis, '52) and special procedures are necessary to determine xanthurenic acid. Since clinical tests for vitamin B_6 deficiency may be required only infrequently, a test that utilizes standard clinical methods would be preferable. The animal studies reported here suggest that such a test is available.

[1] This study was part of a Northeast Regional Project (NE-37, Relationships between protein and other selected nutrients and their metabolism and utilization); a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds.

[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Agricultural Biochemistry, New Brunswick.

The biochemical test proposed for vitamin B_6 deficiency is a curative test in which response to administration of the pure vitamin is measured. From the clinical viewpoint such a test would have the advantage of combining vitamin therapy with the diagnostic test. Curative tests also are preferable to single measures of tissue concentrations because they eliminate individual variation in tissue concentrations. A rapid response is necessary, however, to minimize the effects of other variables during the treatment. In the experiments reported here the response of serum glutamic-oxalacetic transaminase (GOT) in vitamin B_6-deficient rats was found to occur largely within 24 hrs. after feeding. It had previously been shown that transaminases in other tissues and whole blood of vitamin B_6-deficient animals respond by increasing activity after administration of the vitamin (Marsh et al., '55). However, since the method used for measuring transaminase activity was reported to be unsatisfactory for quantitative studies in whole blood, the method of Steinberg, Baldwin and Ostrow ('56) for transaminase in serum was adopted. This method and others (Karmen et al., '54; Reitman and Frankel, '57) are now widely used in clinical laboratories for the estimation of tissue damage caused by myocardial infarction and other degenerative diseases. The findings reported here on effects of vitamin B_6 nutrition on serum GOT are pertinent to the interpretation of this measure of tissue degeneration.

Since completion of the studies described here, Brin and Tai ('58) and Hsu et al. ('58) have reported that the activities of serum transaminases are reduced in vitamin B_6-deficient rats. Ranke et al. ('58) reported an increase in serum GOT activity of aged persons following administration of pyridoxine. Sass and Murphy ('58) have found that the activity of whole blood transaminase is reduced by isonicotinyl hydrazide therapy and restored by administration of pyridoxine.

EXPERIMENTAL

In 4 experiments weanling rats were maintained on a purified diet deficient in vitamin B_6 for 5 to 6 weeks, then supplemented with pyridoxine. Blood and urine samples were taken at the end of the depletion period, and one day and 8 days after supplementation. The blood serums were analyzed for GOT and the urines were analyzed for xanthurenic acid. In two of the 4 experiments the urine collections were preceded by oral doses of tryptophan. Xanthurenic acid excretion following a dose of tryptophan was used to confirm the presence of vitamin B_6 deficiency in the test animals. Control animals were fed the purified diet plus pyridoxine, but the food intakes were restricted to keep the controls at approximately the same body weights as the deficient animals. Details of the experimental procedure follow.

In each experiment, male Wistar-strain rats, 3 to 5 weeks of age, were placed in individual metabolism cages. They were fed a diet of the following percentage composition for 5 to 6 weeks: vitamin-free casein 18.0, coconut oil 5.0, sucrose 70.8, Jones-Foster salt mixture 4.0, vitamin mixture [3] 2.2. Coconut oil was used in the diet to avoid the sparing action of essential fatty acids on pyridoxine requirements (Sherman, '50). Near the end of the depletion period, a 24-hour urine sample was collected. A few days later each animal was anesthetized with ether and approximately 0.7 ml blood was withdrawn by heart puncture. Each animal was then given 0.1 mg of pyridoxine-hydrochloride in water and the diet was supplemented with 0.0022% of pyridoxine for the remainder of the experiment. Urine was collected again during the 24-hour period following the blood sampling and a second blood sample was taken 24 hours after the first blood sample. A third collection of urine and blood was made one week later.

Control animals were treated in the same manner, except that the diet was supplemented with 0.0022% pyridoxine

[3] Nutritional Biochemicals Corp., vitamin diet fortification mixture in dextrose, pyridoxine omitted.

throughout the experiment and the total food intake was restricted to keep the control rats at approximately the same body weight as the test group. In three of the experiments a few additional animals were fed the supplemented diet ad libitum. Because of the small numbers of these animals, the ad libitum data from the three experiments were combined for statistical analysis. In experiments 3 and 4, 30 mg of L-tryptophan was added to the ration of all animals at the beginning of each urine collection.

Serum GOT was measured by the method of Steinberg, Baldwin and Ostrow ('56). The quantities of reagents were reduced by a factor of 10, except that the quantity of DPNH was reduced only by a factor of 5 and 0.03 ml of serum was used. Narrow cells (1.5 $\times$ 10 mm light path) were used in the Beckman DU spectrophotometer. Xanthurenic acid in urine was determined by the method of Rosen, Lowy, and Sprince ('51). Statistical analyses of differences between the means of each group were made by the *t*-test with unpaired data and with $P < 0.01$ as the criterion of significance.

RESULTS

Rats on the deficient diet were retarded in growth and many developed acrodynia. These symptoms were alleviated when the diet was supplemented with pyridoxine. Average xanthurenic acid excretions and serum transaminase activities for each experiment are given in table 1.

The xanthurenic acid excretion of the depleted animals was not significantly higher than that of the control group except when a tryptophan load preceded the urine collection. The decrease in xanthurenic acid excretion by the test group after feeding pyridoxine was, likewise, usually not significant except where the tryptophan load test was used. With the tryptophan load test, the xanthurenic acid excretions of the depleted group were significantly higher than those of the control group and they were reduced significantly during the first 24 hours after feeding pyridoxine. There was no significant change in xanthu-

TABLE 1

Average xanthurenic acid excretions and serum transaminase (GOT) activities of vitamin B_6-deficient and control rats before and after supplementation with vitamin B_6

EXPERIMENT	GROUP	NO. RATS	XANTHURENIC ACID (μG/24 HR.)			SERUM TRANSAMINASE (UNITS/ML)		
			Depleted	Plus vitamin B_6		Depleted	Plus vitamin B_6	
				1 day	8 days		1 day	8 days
1	Deficient	5–11	150 ± 44 [1]	113 ± 32	236 ± 23	4 ± 2.7 [1]	37 ± 8.0	69 ± 4.1
	Control	6	166 ± 41	99 ± 13	215 ± 55	104 ± 8.5	67 ± 14.9	78 ± 6.0
2	Deficient	2–7	248 ± 32	64 ± 16	144 ± 31	19 ± 3.6	65 ± 16.6	62 ± 5.2
	Control	5–6	96 ± 29	100 ± 29	82 ± 27	98 ± 8.5	90 ± 12.3	78 ± 11.0
3 (with trypto-	Deficient	7–8	1728 ± 498	123 ± 16	218 ± 25	26 ± 2.5	70 ± 5.9	75 ± 2.6
phan load test)	Control	6	198 ± 37	270 ± 50	115 ± 6	85 ± 5.6	79 ± 5.9	78 ± 9.6
4 (with trypto-	Deficient	6–9	2795 ± 704	354 ± 64	—	21 ± 1.3	57 ± 6.5	77 ± 5.2
phan load test)	Control	6	213 ± 54	376 ± 117	—	95 ± 14.7	70 ± 3.0	64 ± 3.0
Averages:	Deficient					17.5	57.2	70.8
	Control					95.5	76.5	74.5
	Ad libitum	9–10				68 ± 6.3	69 ± 4.0	52 ± 3.4

[1] Standard error of the mean.

renic acid excretions by the control animals. These findings confirm the existence of vitamin B_6-deficiency in the test animals and further show that the vitamin is rapidly incorporated into the enzyme system required for normal tryptophan metabolism.

The serum glutamic-oxalacetic transaminase (GOT) activity of the deficient animals was significantly lower than that of the control animals in each of the 4 experiments. In each experiment there was a significant increase in GOT activity of the deficient animals 24 hours after feeding pyridoxine. After feeding the vitamin supplement, there was no longer any significant difference between the GOT activities of the test and control animals. There was no significant difference between GOT activities of the control animals before and 24 hours after feeding extra pyridoxine. Since rats deficient in vitamin B_6 consistently had lower serum GOT activities than either control animals or the test animals after supplementation with the vitamin, it may be concluded that a deficiency of vitamin B_6 reduced the activity of this enzyme in blood serum. This finding is in agreement with the recently reported observations of other workers cited above.

The increase in serum GOT after feeding pyridoxine was about the same when tryptophan was fed (experiments 3 and 4) as when the tryptophan load test was not used (experiments 1 and 2). Since this finding suggests that the tryptophan load does not affect the serum GOT test, the GOT data from experiments 1 and 2 have been averaged with those from experiments 3 and 4. These averages (table 1) show that the GOT activity of the control animals was more than 5 times as high as that of the deficient animals and also that the average GOT activity of the deficient animals was tripled 24 hours after feeding pyridoxine. After 8 days of supplementation with the vitamin the average GOT activity was 4 times as high as during deficiency.

The restricted intake controls, meanwhile, showed small but non-significant decreases in GOT activity, which gave them

nearly the same average value at the end of the experiment as the repleted test animals. The rats fed the supplemented diet ad libitum also showed a decrease in transaminase activity ($P < 0.05$), but with these much heavier rats the decrease did not take place until the third bleeding. Blood dilution, and possibly indirect effects of anesthesia, may have contributed to the decreases observed in serum transaminase activities of the control animals. Heart damage, as from the bleeding operation, would be expected to cause an increase, rather than a decrease, in serum transaminase (Steinberg and Ostrow, '55).

CONCLUSIONS

The activity of glutamic-oxalacetic transaminase in blood serum was considerably reduced in rats which were deficient in vitamin B_6.

Vitamin B_6 was incorporated into glutamic-oxalacetic transaminase, and also into an enzyme system involved in tryptophan metabolism, within 24 hours after feeding the vitamin.

ACKNOWLEDGMENTS

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THE EFFECT OF METHIONINE DEFICIENCY ON NITROGEN ABSORPTION FROM THE INTESTINAL TRACT OF CHICKENS [1,2]

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INTRODUCTION

There have been many reports that amino acid deficiencies will cause a decrease in the activity of various enzymes. Most of these studies have dealt with special organs, particularly the liver, and little or no information is available on the effect of an amino acid deficiency on protein digestion and absorption in the intestinal tract.

A slightly different approach was used in the present work in that some of the enzymes of digestion (and possibly of absorption), were studied by means of digestibility studies on total nitrogen of the diet. Samples of the contents from various levels of the gastrointestinal tract were tested instead of the feces so that it was possible to follow digestion and absorption of protein nitrogen as the food passed down the tract. This approach not only offers a more complete basis for com-

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parison but yielded information on the little studied processes of digestion and absorption in the chick.

Several authors have used the chromic oxide method to measure digestibility by the chick but in no case was nitrogen specifically measured due to the obvious difficulty caused by the simultaneous voiding of urine and feces. Thus Dansky and Hill ('52) and Mueller ('56) measured only the percentage of chromic oxide in the total dry matter of the droppings and Yoshida and Morimoto ('57) measured the loss of total reducing sugars.

METHODS

Digestion estimations were made by the chromic oxide indicator method described by Schürch et al. ('50). The method involves the incorporation of a known amount of a completely indigestible, non-absorbable and inert material, Cr_2O_3, in the feed. By determining the ratios of Cr_2O_3 and nitrogen in the feed and contents of the tract, an apparent coefficient of digestion or absorption for the nitrogen of the feed can be calculated.

Day-old White Leghorn cockerels from a commercial hatchery were used. After 4 days on a commercial mash, one group of 10 chicks was fed a methionine-deficient diet which consisted of Canada field peas, 91%; Briggs et al. ('43) mineral mix, 6.0%; cottonseed oil,[5] 1.8%; vitamin premix in starch, 1%; and fortified cod liver oil,[6] 0.2%. Levels of vitamins used were based on the recommendations of the National Research Council ('50). A second group of 10 chicks received the same diet supplemented with 0.33% of DL-methionine, 0.09% of L-cystine and 0.16% of DL-tryptophan. Previous work (Peterson et al., '44) has shown methionine to be the limiting amino acid in Canada field peas and tryptophan was added only because data (Block and Bolling, '51) suggested the possibility of a borderline level of tryptophan. Supplementation of the peas with 0.33% of methionine gave a calculated value of 0.5% of methionine. The third diet consisted of a commercial

[5] Wesson oil.

[6] Supplied by the Nopco Chemical Co., Harrison, New Jersey.

starter mash which had the following percentage composition: wheat flour middlings 10, yellow corn meal 42.75, fine ground oats 5, alfalfa meal (17% protein) 3, soybean meal (44% protein) 25; meat scraps 2, fish meal 1.5, homogenized condensed fish 1, corn distillers dried solubles 3, riboflavin supplement 0.4, dried whey fortified with riboflavin 3, dicalcium phosphate 2, ground limestone 1, iodized salt 0.25, vitamin D supplement, vitamin B_{12} and antibiotic supplement and manganese sulfate. This ration carried a guaranteed analysis of at least 20% protein (by analysis 21.9%) at least 3% fat and not over 5% fiber. By calculation the pea diets should have contained 21.3% protein, 3.1% fat and 5.5% fiber. Actually, the hulls of the peas did not grind readily and were largely discarded so that the protein content, by analysis, was 25.0% and the fat presumably raised and the fiber lowered. These differences were considered unimportant, based on general experience with chicks, and on the fact that the primary comparison was between the methionine-supplemented and unsupplemented pea diets.

Chicks were fed these diets ad libitum in an electrically heated brooder with raised screen floors. After three months, the chicks were fed the diets mixed with indicator. In order to present homogeneous diets to the chicks, it was necessary to grind the diets further until they were about as finely dispersed as the chromic oxide. Diets containing 1% of the indicator were fed for a period of 30 to 40 hours to chicks which had been without feed for 22 hours. The chicks were deprived of feed to allow emptying of the tract, and feeding the test diets for 30 to 40 hours insured complete equilibrium of the diets in the intestinal tracts when the animals were sacrificed. Tests by the authors have shown that chromic oxide can appear in the feces of chicks within two and one-half hours after feeding.

Chicks were sacrificed and representative samples of the contents of the crop, gizzard, and intestine dried overnight at 65°C. After drying, the materials were finely ground with a mortar and pestle and stored in tightly-stoppered test tubes.

Samples were taken from 4 different levels of the intestine. The duodenal loop comprised the first quarter and the remainder of the small intestine, down to the union with the cecum, was divided evenly into the other three portions. Each of the 4 divisions was cut in half and the contents simply squeezed out, starting at each end, by gently pressing with the thumb and forefinger. The gastrointestinal tracts of all groups were full and large quantities of material were available. The part of the intestine below the cecum, the large intestine or rectum, represents only about 7% of the entire length of the intestine and its contents were disregarded. The crop and gizzard were also sampled but results were highly erratic, due, it is believed, to variable regurgitation of food.

Nitrogen was determined by the microKjeldahl method of Ma and Zuazaga ('42) and chromic oxide by the method of Schürch et al. ('50) except that the dichromate color was read at 375 mμ as recommended by Dansky and Hill ('52).

RESULTS AND DISCUSSION

Growth. After three months on the diets, chicks of the amino acid-supplemented groups had an average weight of 1200 gm and compared well in general appearance with the starter mash group which weighed 1400 gm. Although there may have been a slight deficiency of some factor(s) the birds were essentially normal. Chicks on the Canada field peas unsupplemented with amino acids weighed only 480 gm. Further supplementation of the pea diet with a group of amino acids — histidine, glycine, leucine, phenylalanine, and valine — was without beneficial effect on growth.

Nitrogen absorption. Table 1 shows that in all groups of chicks the crop contents had roughly the same composition with respect to nitrogen and chromic oxide as did the food, indicating relatively little digestion, absorption, or selective movement of the contents. In the gizzard there was a drop in both nitrogen and chromic oxide. In the case of the starter mash group the gizzard contained solid particles which apparently had accumulated during the three-month preliminary

TABLE 1

Apparent absorption coefficients in normal and methionine-deficient chickens

PORTION OF G. I. TRACT	STARTER MASH CONTROL			SUPPLEMENTED PEA DIET			UNSUPPLEMENTED PEA DIET		
	N	Cr_2O_3	A.C. [1]	N	Cr_2O_3	A.C. [1]	N	Cr_2O_3	A.C. [1]
	% [2]	% [2]		% [2]	% [2]		% [2]	% [2]	
Diet	3.5	1.10		4.0	1.00		4.0	1.00	
Crop	3.7	1.10	− 6	3.5	0.87	− 1	3.7	0.94	+ 2
Gizzard	2.2	0.67	− 3	2.5	0.94	+ 34	2.3	0.78	+ 26
Duodenal loop	8.2	0.93	− 176	7.6	0.79	− 159	6.0	0.84	− 79
2nd quarter	4.5	2.3	+ 39	3.4	2.2	+ 61	2.2	2.2	+ 75
3rd quarter	2.9	3.5	+ 74	2.3	3.3	+ 83	1.7	3.1	+ 86
4th quarter	2.2	4.1	+ 83	2.2	4.2	+ 87	1.8	4.6	+ 90

[1] A.C. = Absorption coefficient $= \left(1 - \frac{\%\ Cr_2O_3 \text{ in feed}}{\%\ Cr_2O_3 \text{ in tract}} \times \frac{\%\ \text{nitrogen in tract}}{\%\ \text{nitrogen in feed}}\right) \times 100.$

Absorption coefficients in the table were calculated from the average N and Cr_2O_3 values given.

[2] Average of values from 10 chicks.

period before the ration was finely ground. These served to lower both nitrogen and chromic oxide contents still further. The gizzard is not an organ of absorption (Sturkie, '54) and the drop in nitrogen, and to some extent chromic oxide, probably reflects the selective movement of soluble, partly digested protein and finely dispersed chromic oxide. The strong muscular contractions of the gizzard tend to pass the fluid and finely dispersed fraction first, as evidenced by the fact that the gizzard contents contained less water than the crop, which might be interpreted as absorption. No absorption was indicated in birds fed the starter mash, presumably because the protein was more resistant to solubilization.

Pepsin is always present in the contents of the gizzard (Sturkie, '54) coming from the proventriculus. This latter organ was not tested because of the small amount of food present and the short time it remains there. Gizzard content has a pH of 2 to 3 and significant peptic digestion undoubtedly occurs, resulting in some solution of food protein and selective passage down the tract. While it is generally agreed that the chief function of the gizzard is in grinding the food, its role in peptic digestion should not be minimized. Below the gizzard the contents have a pH of 5.8 to 6.4, so that trypsin and similar proteolytic enzymes become active.

In the duodenum, the high nitrogen concentration, lower chromic oxide and negative absorption coefficients all indicate a considerable secretion of protein into the tract and mask any possible absorption detectable by this method. This finding is in agreement with the well-known fact that the pancreas and intestinal mucosa secrete enzymes into the duodenum. The nitrogen content and, therefore, the enzyme secretion of the duodenal contents of the methionine-deficient group was definitely lower than that of the other groups. However, this had no noticeable influence on protein digestion, since the absorption coefficients further down the tract were highest for this group. These findings tie in rather well with the suggestion of Nasset ('57) that there is sufficient secretion of protein into the tract to present an almost uniform pattern

of amino acids for absorption and that the presence of food is in some way stimulatory to this secretion.

Absorption was first apparent in the portion of the small intestine just below the duodenal loop, where the amount of absorption was also greatest. Absorption continued, at decreasing rates, down to the cecum. Contrary to expectations, the apparent absorption coefficients were highest in the methionine-deficient group, followed by the methionine-supplemented group and lastly by the starter mash controls. The chromic oxide values for all three groups were about the same, the higher absorption coefficients being due to the lower concentrations of nitrogen remaining.

Although the initial assumption had been that a copious secretion of digestive enzymes was a prerequisite for rapid absorption, it is conceivable that the lower secretion in itself contributed to the high absorption simply because it provided less nitrogen to be reabsorbed. It is interesting that the nutritive quality of the rations, as indicated by growth rates, is also reflected in the secretion of nitrogen, presumably enzymes, into the duodenal loop.

The change in going from the second to the third quarter of the small intestine shows a continuation of the digestive and absorptive process. The extent of absorption was probably largely guided by the amount of nitrogen which remained to be absorbed. Thus, in the chicks on starter mash, the concentration of nitrogen was about twice that of the methionine-deficient birds. Obviously the latter birds could show relatively little additional absorption. The fact that chromic oxide concentrations increased markedly indicated absorption of all types of nutrients, with the chicks on starter mash showing the greatest change.

In the 4th quarter of the intestine, less significant changes were noted, indicating that the major part of digestion and absorption had been accomplished before the food had gone this far.

Statistical analyses of absorption coefficients reveal several significant differences between the three groups, with the

differences narrowing as absorption approached completion. Comparison of the methionine-supplemented and unsupplemented pea diets shows a significantly higher absorption coefficient in the deficient group in the second quarter, with the differences in the third and 4th quarters approaching significance. Differences between the starter mash control and deficient groups were highly significant in all three levels. The significantly higher absorption coefficient in the deficient group clearly indicates that methionine deficiency did not result in a decreased ability to digest and absorb the protein nitrogen which was consumed. A consideration of the methionine-supplemented and starter mash controls reveals borderline significance in the second quarter difference, definite significance in the third quarter, but an insignificant difference in the 4th and final quarter, indicating slightly more rapid initial absorption but no overall difference. The lower absorption coefficients of the starter mash group at the upper levels in the tract might be due to a lower digestibility of this diet which consisted of a variety of vegetable and animal proteins, in contrast to the other two groups of chicks which received field peas as the only food.

In going from the third to the 4th quarter, the absorption coefficient rose 9% from 74 to 83%, in the starter mash group, which was more than double the gain in the other two groups, but it still brought the total absorption to only 83%. This finding raises the question of whether high consumption of a good ration may not limit the overall digestibility of the ration due to the speed with which nutrients must pass through the digestive tract. Dansky and Hill ('52) indicate that there may be such a relation but Yoshida and Morimoto ('57) failed to corroborate this finding. In this connection, Sturkie ('54) stated that food will pass through the digestive tract of birds in a few hours when fed ad libitum but that it takes about 24 hours for a full crop to empty when chicks are taken off feed.

Absorption of nitrogen by the chick, using the chromic oxide indicator method, ranged from 83 to 90%, depending upon the

type of ration fed. These values are higher than those reported by Dansky and Hill ('52) and by Mueller ('56) for total dry matter, which is logical because of the presence of various undigestible constituents, especially fiber. They are slightly lower than the values of Yoshida and Morimoto ('57) for carbohydrates. It should be pointed out, however, that in the present studies any absorption below the cecum was not measured so that the values reported may be minima. The results clearly indicate that the methionine-deficient chicks are not limited in their ability to digest and absorb the relatively low amount of protein which they consume.

SUMMARY

Protein absorption was determined in various levels of the digestive tract of chicks on adequate and methionine-deficient diets using the chromic oxide indicator method.

No digestion or absorption was detected in the crop. The nitrogen concentration of gizzard contents was less than that of the feed suggesting digestion due to the presence of enzymes secreted by the proventriculus, solubilization, and selective movement of fluid from the gizzard. There was marked secretion of nitrogenous compounds, presumably largely enzymes, into the duodenal loop, thus obscuring any possible absorption of protein in this region. Nitrogen absorption was evident throughout the entire length of the small intestine below the duodenal loop with the portion just below the duodenum being most active.

Methionine-deficient chicks are fully able to digest and absorb the protein in the limited amount of diet consumed.

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THE EFFECT OF α-TOCOPHEROL, α-TOCOPHERYLHYDROQUINONE AND THEIR ESTERS ON EXPERIMENTAL MUSCULAR DYSTROPHY IN THE RAT[1]

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In 1940 Mackenzie and McCollum showed that the muscular dystrophy of rabbits, first produced experimentally by Goettsch and Pappenheimer ('31) could be prevented or cured by the oral administration of α-tocopherol. When the vitamin was injected subcutaneously neither the intense creatinuria nor the muscle wasting was arrested, although the life of the dystrophic animal was prolonged (Mackenzie and McCollum, '41).

Some 10 years later Mackenzie, Rosenkrantz, Ulick and Milhorat ('50) discovered that the hydroquinone of α-tocopherol also cured acute muscular dystrophy in the rabbit. The activity of the hydroquinone was greatly enhanced when it was given intravenously, but it was not stored in the body to any appreciable extent. Nevertheless a small intravenous dose of hydroquinone was just as effective in alleviating creatinuria as an equivalent dose of α-tocopherol administered by stomach tube. Because of the great susceptibility of α-tocopherylhydroquinone to autooxidation, Farber, Mackenzie, Rosenkrantz and Milhorat ('51) investigated the biological activity of several esters and found that both the disuccinate and diace-

[1] These studies were made possible by a grant from the Muscular Dystrophy Associations of America, Inc.

tate, while less potent than the hydroquinone on a molar basis, caused a fall in the creatine excretion of dystrophic rabbits.

West and Mason ('55) confirmed the antidystrophic activity of α-tocopherylhydroquinone, using hamsters as the test animal. However, their oral preparation, stabilized with butylhydroxyanisole, had only one-third of the antidystrophy activity of α-tocopherol. Subsequently, Harris and Mason ('56) in a superbly designed experiment fed α-tocopherylhydroquinone to children with muscular dystrophy and found it to be without effect on creatine excretion, serum aldolase or muscle function.

Recently, in the course of experiments on muscular dystrophy in the rat, we observed that the intravenous administration of α-tocopherylhydroquinone disuccinate had little or no effect on creatine excretion. Because of the implications of this finding with respect to both experimental and human dystrophy, we felt obliged to examine the activity of the free hydroquinone and of α-tocopherol itself in our animals. This seemed particularly desirable in view of the doubt expressed concerning the ability of α-tocopherol to cure the chronic muscle lesions in adult rats (Pappenheimer, Goettsch, Ritzmann and Shogoleff, '56).

MATERIALS AND METHODS

Male and female Sprague-Dawley rats weighing 50 to 65 gm were placed on a vitamin E-deficient diet and housed in individual cages with raised screen bottoms. The composition of the basal diet is shown in table 1. In order to exclude the antioxidants that are now added routinely to commercial lard, the lard was prepared in the laboratory as follows. Forty pounds of fresh leaf lard were heated in an open aluminum pan at a temperature not exceeding 80°. The melted material was immediately strained through cheese cloth and stored in glass containers at 4° until added to the diet. At no time did this lard develop a rancid odor. Fresh diet was prepared weekly and the uneaten portion was replaced at least twice a week.

The rabbits used for testing compounds for antidystrophy activity weighed approximately 400 gm when they were placed on the dystrophy producing diet 13 of Goettsch and Pappenheimer ('31). They were housed in individual stainless steel metabolism cages.

α-Tocopherylhydroquinone was prepared by the hydrogenation of *d*- or *d,l*-α-tocopherylquinone.[2] Approximately 500 mg of α-tocopherylquinone, containing less than 1% of reducing

TABLE 1

Basal diet

CONSTITUENT	AMOUNT
	gm
Casein [1]	200
Sucrose	668
Lard [2]	100
Salt mixture [3]	20
Choline chloride	2
Vitamin mixture [4]	10

[1] Nutritional Biochemicals Corp., Vitamin Free.

[2] Pork leaf lard rendered at 80°C in the laboratory.

[3] Hubbell, Mendel and Wakeman ('37), prepared by Nutritional Biochem. Corp.

[4] The vitamin mixture was composed of 10 mg thiamine·HCl, 10 mg riboflavin, 10 mg pyridoxine·HCl, 10 mg nicotinamide, 50 mg calcium pantothenate, 0.1 mg biotin, 0.1 mg vitamin B_{12}, 1.0 mg folic acid, 10 mg *p*-aminobenzoic acid, 100 mg *m*-inositol, and pulverized sucrose to 10 gm. 2.5 mg of vitamin K (Menadione) and 10 drops of oleum percomorphum (Mead) were added to each kilogram of diet.

materials, were dissolved in 5 ml of propylene glycol diluted with 10% absolute ethanol and this solution was added to a 450-ml Parr pressure bottle containing 500 mg of 5% palladium on $CaCO_3$.[3] The bottle was filled with hydrogen at a pressure of 45 lb. per sq. in. and was shaken for three hours. Approximately 2 ml of the mixture was then removed and centrifuged for 8 min. in a table model centrifuge. An aliquot of the clear supernatant fluid containing the hydroquinone was withdrawn and diluted with absolute ethanol to give a con-

[2] We are indebted to the Distillation Products Industries for the *d* isomer and to Dr. Milton Farber and Dr. A. T. Milhorat for the racemic mixture of α-tocopherylquinone as well as for our supply of *d,l*-α-tocopherylhydroquinone disuccinate.

[3] Baker and Company.

centration of 30 to 60 μg per 8 ml. The reducing material in this solution was measured in a Coleman, Jr. spectrophotometer by the method of Emmerie and Engel ('38). Alpha-tocopherol dissolved in ethanol was used as the standard for calculating the concentration of α-tocopherylhydroquinone in the undiluted reaction mixture. The yield of hydroquinone was consistently 90% or better. However, the rapid oxidation of the hydroquinone to the quinone, even when the solution was stored in the refrigerator under nitrogen, necessitated the preparation of a fresh sample daily. This was accomplished by rehydrogenating the material that remained in the Parr bottle and reanalyzing the supernatant fraction.

For purposes of injection, aliquots of the centrifuged supernatant solution described above were removed in 0.25-ml tuberculin syringes and immediately given to the test animals. The preparation of rats for repeated injections in the tail vein has been described in an earlier paper (Mackenzie and Mackenzie, '53). Rabbits were injected in the marginal ear vein. Analysis showed that there was essentially no oxidation of the hydroquinone in the syringes within the time required to inject the test animals.

d,l-α-Tocopherylhydroquinone disuccinate was injected in a phosphate buffer, adjusted to pH 7.2, which contained 8.1 mg of disuccinate per ml. *d,l*-α-Tocopherol [4] and *d,l*-α-tocopherol acetate [4] were injected in a propylene glycol-10% ethanol solution containing 1.4 mg of the vitamin per 0.05 ml. For oral administration, the *d,l*-α-tocopherol acetate was dissolved in stripped olive oil [5] to give a solution containing 5 mg per 0.05 ml.

Creatine and creatinine were measured in 24-hour urine samples by the method of Folin ('14). To avoid contamination of the urine with the purified diet, which increases the Jaffé reaction significantly after autoclaving, food was withheld from the rats during the 24-hour urine collection period. This precaution was not necessary in the rabbit assays inasmuch

[4] Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Distillation Products Industries, Rochester, New York.

as the small quantities of diet 13 present in the urine did not contribute appreciably to the color reaction.

Muscles were prepared for histological examination by fixing in Zenker-formol solution and staining with hematoxylin and eosin. The following muscles were sectioned; triceps brachii, semitendinous, semimembranosus, and vastus lateralis.

RESULTS

The vitamin E-deficient diet, which is also free of commercial antioxidants, produced a decrease in the growth rate of

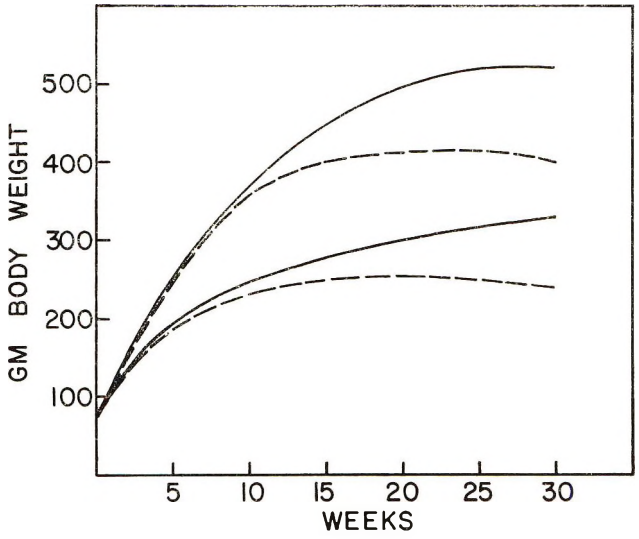

Fig. 1 Reduction of growth rate in vitamin E-deficient rats. The upper curves are for male rats and the lower curves are for female rats. The solid lines represent animals supplemented with 5 mg of *d,l*-α-tocopherol acetate twice a week and the broken lines represent the deficient animals.

both male and female rats as compared with the growth of control animals fed 5 mg of α-tocopherol acetate twice a week. This growth effect of vitamin E deficiency became apparent between the 10th to 15th weeks (fig. 1) of the experiment, and by the end of the 20th week the control rats weighed 20% more than the deficient animals of the same sex.

After 4 months on the diet the creatine excretion of the vitamin E-deficient animals was 5 to 8 times greater than the control level. This creatinuria persisted throughout the remainder of the experiment (table 2). Histological examination of the skeletal muscles of the deficient rats at 20 to 25 weeks revealed the presence of lesions which were more extensive than we have seen hitherto in an uncomplicated vitamin E deficiency in this species. Instead of only one small area of nuclear and monocytic accumulation in several low power fields (Mackenzie, '53), most fields contained three or 4 lesions and many of these involved extended segments of the muscle fibers (fig. 2). By 30 weeks the lesions were even more exten-

TABLE 2

Creatine and creatinine excretion in vitamin E deficient rats

Creatine and creatinine excretion per 24 hours expressed as milligrams per 100 gm of body weight. Each figure represents the average value obtained from 6 to 12 animals.

SUPPLEMENT	EXCRETION	MONTHS ON DIET				
		4	5	6	7	9
None	Creatine	2.1	3.1	2.5	2.8	2.5
	Creatinine	2.3	2.2	2.8	2.4	2.2
Vitamin E	Creatine	0.4		0.3		
	Creatinine	2.5		2.8		

sive and they frequently approached the severe muscle damage first produced by us when a deficiency of vitamin B_6 was superimposed on vitamin E deficiency (Mackenzie, '53). However, the present experimental diet (table 1) contained adequate amounts of all of the known vitamins except vitamin E and no symptoms attributable to any other vitamin deficiency were observed. Collectively, the symptoms observed in these animals, i.e., depressed growth, creatinuria and muscle damage, indicated that they had a relatively severe degree of muscular dystrophy, and it appeared, therefore, that they would be well suited for investigating the response of this species to the intravenous injection of compounds known to be effective in curing the fulminating muscle degeneration in vitamin E deficient rabbits.

In preliminary experiments it was found that injection of the disuccinate of α-tocopheryllhydroquinone had very little effect on the creatinuria of the dystrophic rats even after daily administration for two weeks. It seemed necessary therefore to determine whether or not the dystrophic rats would respond to the oral administration of vitamin E. As shown in table 3, a single 5 mg dose of α-tocopherol acetate

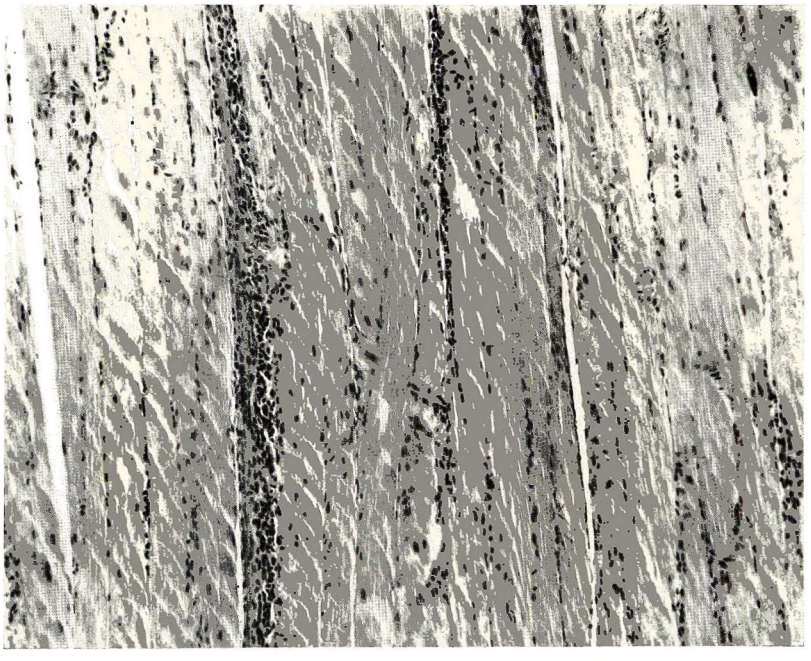

Fig. 2 Lesions in skeletal muscle of a rat fed the vitamin E-deficient diet for 23 weeks. × 150.

markedly reduced the excretion of creatine within three days. After 6 days, the creatine excretion was essentially normal. Examination of the skeletal muscles at the end of two weeks, when each rat had received a total of 20 mg of the acetate, showed that for practical purposes the dystrophy had been cured. No lesions were found in the 4 muscles taken from each of three rats, while in the remaining case only two or three small lesions were present in two of the 4 sections.

Since the preceding experiment clearly demonstrated the ability of the dystrophic rat to respond to the oral administration of vitamin E, the disuccinate preparation, which had been inactive in the rats, was tested in dystrophic rabbits. Although its potency was less than that of the free hydroquinone, it was shown nevertheless to possess definite antidystrophy activity in the rabbit (table 4).

The question now arose as to whether or not dystrophic rats would respond to the intravenous administration of a compound that was of proven activity when given orally. To answer this, dystrophic rats maintained on the deficient diet for 25 weeks were injected daily with either α-tocopherol or

TABLE 3

Response of dystrophic rats to oral α-tocopherol acetate

After the experimental animals had been fed the vitamin E diet for 23 weeks they were fed 5 mg of the acetate twice a week beginning at 0 days. The urinary creatine excreted in 24 hours is expressed as milligrams per 100 gm body weight.

SEX	CREATINE EXCRETION				MUSCLE LESIONS
	0 days	3 days	6 days	11 days	14 DAYS
Male	2.0	0.3	0.2	0.3	0
Male	4.2	1.1	0.9		±
Female	3.4	0.4	0.3		0
Female	2.5		0.6	0.2	0

α-tocopherol acetate for two weeks. The free tocopherol produced a large drop in creatine excretion within three days and at the end of one week the urinary excretion of creatine was normal (fig. 3). The acetate, on the other hand, gave a much slower response and even after two weeks the creatine excretion had not reached the normal level. Furthermore, at the end of two weeks the muscles examined from animals treated with α-tocopherol acetate contained two to 5 lesions per section, whereas the free tocopherol during the same time had completely cured the muscle lesions.

TABLE 4

Response of dystrophic rabbits to intravenous α-tocopherylhydroquinone disuccinate and α-tocopherylhydroquinone

Young vitamin E-deficient rabbits weighing approximately 1 kg were injected with a single dose of the test compound when their daily creatine excretion had exceeded 40 mg for two days.

COMPOUND	α-TOCOPHEROL EQUIVALENCE	NO. OF RABBITS	CREATINE EXCRETION AT TIME OF SUPPLEMENT	MAXIMAL DROP IN CREATINE EXCRETION [1]	DURATION OF LOWERED CREATINE EXCRETION
	mg		*mg*	%	*days*
α-Tocopherylhydroquinone disuccinate [2]	40	9	65	63 ± 5	4.0 ± 5
d,l-α-Tocopherylhydroquinone	8	6	62	83 ± 3.8	6.7 ± 0.7
d,l-α-Tocopherol acetate [3]	9	6	70	91 ± 1.3	15.0 ± 1.5

[1] $\frac{\text{Maximal drop in creatine (mg)}}{\text{Creatine (mg) at time of supplement}} \times 100.$

[2] Four animals received the *d,l* compound and 5 received the *d* compound. No difference was observed in the response to the two preparations, which were injected as the disodium salt in 3 ml of phosphate buffer.

[3] Administered by stomach tube.

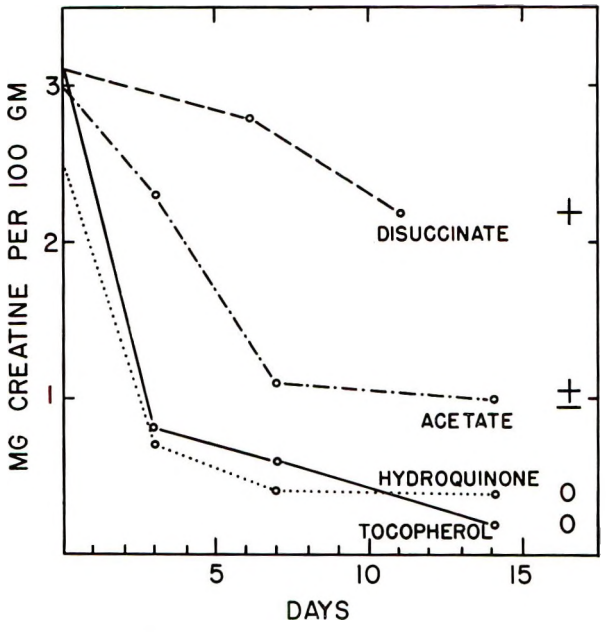

Fig. 3 The response of dystrophic rats to α-tocopherol and its derivatives. Each compound was injected by tail vein daily for two weeks at the following levels; 8 mg of *d,l*-α-tocopherylhydroquinone disuccinate, 1.4 mg of *d,l*-α-tocopherol acetate, 1.8 mg of *d*, or *d,l*-α-tocopherylhydroquinone, and 1.4 mg of *d,l*-α-tocopherol. Each group consisted of 5 or 6 rats fed the vitamin E-deficient diet for 25 to 30 weeks, except the hydroquinone animals which were not supplemented until the end of 35 weeks. The muscle lesions of unsupplemented control rats were scored as ++ to +++. The lesions present in the muscles of the injected rats after 14 days of treatment are indicated at the right of the figure.

In view of the foregoing results, α-tocopherylhydroquinone was prepared daily [6] and its antidystrophy activity in rats was compared with the disuccinate. The α-tocopherylhydroquinone was injected at a molar level that approximately equalled the α-tocopherol given in the previous experiment, whereas the disuccinate was injected at 4 times the molar level of the tocopherol. The disuccinate produced only a slight fall in creatine excretion (fig. 3) and at the end of two weeks the muscle lesions had been reduced by no more than 50%.

[6] The preparation and chemical analysis of many of the samples of α-tocopherylhydroquinone were carried out by Mr. Jerome V. Holland, who was supported by a Lederle Laboratories Medical Student Research Fellowship.

The free hydroquinone, on the other hand, reduced the creatine excretion at a rate comparable to that obtained with α-tocopherol. Furthermore, the hydroquinone was equally effective in curing the muscle lesions.

DISCUSSION

The experiments reported in this paper show that a relatively severe muscular dystrophy in the rat can be cured by α-tocopherol and by α-tocopherylhydroquinone. Furthermore, they show that, in intravenous therapy, these compounds are much more effective than their respective acetyl and succinyl esters. Possibly the rate of hydrolysis of the esters when given intravenously is too slow or, alternatively, they may be excreted or metabolized at a faster rate than the free alcohols. Whatever the explanation, the disuccinate of α-tocopherol is a poor substitute for the parent compound. These results have implications with regard to vitamin E therapy in man in view of the important observation of Nitowsky, Gordon and Tildon ('56) that children with cystic fibrosis of the pancreas have low plasma tocopherol levels and creatinuria. Furthermore, Oppenheimer ('56) has described muscle lesions in a case of cystic fibrosis of the pancreas that were indistinguishable from the lesions seen in experimental dystrophy. When a rapid response is essential in such cases it would appear from our results that free tocopherol may be superior to its esters. In this connection Nitowsky, Gordon and Tildon ('56) also observed a low plasma tocopherol and creatinuria in two children with biliary atresia. In the latter patients, unlike those with cystic fibrosis, tocopherol therapy did not eliminate the creatinuria even when the plasma tocopherol rose to high levels. It is conceivable that the hydroquinone might be effective in reducing the creatinuria in this disease.

In addition to the foregoing considerations, the experiments described in this paper reopen the question as to whether or not α-tocopherylhydroquinone possesses antisterility activity in the female rat. In an earlier publication Mackenzie and Mackenzie ('53) reported that the daily intravenous injection

of 8 mg of α-tocopherylhydroquinone disuccinate failed to prevent foetal reabsorption. Inasmuch as the disuccinate cured dystrophy in the rabbit, presumably because of its hydrolysis to the hydroquinone, we concluded that it and the hydroquinone did not possess antisterility activity as defined by this classical test. It is now evident that this problem will have to be reinvestigated by comparing the antisterility activity of α-tocopherylhydroquinone itself with the activity of α-tocopherol when both compounds are administered in a similar manner. Such a study is in progress.

SUMMARY

A relatively severe state of muscular dystrophy characterized by extensive muscle lesions, creatinuria, and reduced growth was produced in rats fed a vitamin E-deficient diet devoid of antioxidants.

Alpha-tocopherol and related compounds were administered daily to these animals by intravenous injection and the effect on creatine excretion and muscle lesions was determined in a two-week test period.

Both α-tocopherol and α-tocopherylhydroquinone caused a prompt fall in creatine excretion to the normal level and cured the muscle lesions. Alpha-tocopherol acetate produced a slower fall in creatine excretion and residual muscle lesions were present at the end of the test period. The disuccinate of α-tocopherylhydroquinone exhibited little antidystrophy activity even when injected at 4 times the molar level of the other compounds.

ACKNOWLEDGMENTS

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CALCIUM OXALATE EXCRETION AND HEMATURIA IN VITAMIN B_6-DEFICIENT RATS FED PHTHALYLSULFATHIAZOLE [1]

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During the course of a bioassay for vitamin B_6 in wheat, flour and bread, one male rat developed striking, persistent gross hematuria after 17 days on a basal diet containing phthalylsulfathiazole. This diet was patterned after that of Sarma, Snell and Elvehjem ('46). Microscopic examination of the urine revealed numerous erythrocytes and great numbers of crystals that were positively identified microscopically and chemically as calcium oxalate. The incidence of hematuria increased with time on the deficient diets, and approached 100% in some groups by the end of 7 weeks. Gross hematuria was not observed in a group receiving the basal diet plus 1.5 μg of pyridoxine hydrochloride per gram of diet and animals receiving the vitamin B_6-deficient diet without phthalylsulfathiazole did not develop hematuria but did have oxaluria.

A search of the literature failed to find a report of hematuria or calcium oxalate excretion in the albino rat fed a vitamin B_6-deficient diet with or without phthalylsulfathiazole. Agnew ('51) produced hematuria in the hooded rat (Lister), but not in the white rat (Wistar) by feeding a vitamin B_6-deficient diet. Higgins ('44) found that the administration of large quantities of water-soluble vitamins, including pyri-

[1] Supported by a grant from the Max C. Fleischmann Foundation.

doxine, reduced the toxic lesions produced by the feeding of promin (sodium *p, p'*-diaminodiphenylsulfone-*N, N'*-didextrose sulfonate) to albino rats. Nielsen and Elvehjem ('42) produced folic acid and biotin deficiencies in young rats by feeding an otherwise adequate diet containing 1% of succinylsulfathiazole. No hematuria or kidney damage was reported. In studying pantothenic acid-ascorbic acid relationships, Barboriak and Krehl ('57) fed weanling rats a pantothenic acid-deficient diet containing 2% of phthalylsulfathiazole. No hematuria was reported, and no kidney abnormalities were noted during autopsy examination of vital organs.

EXPERIMENTAL

Sprague-Dawley rats were housed individually in suspended wire-bottom cages in an environmental temperature maintained at 76 to 78°F. Weekly weights were recorded.

The vitamin B_6-deficient basal diet contained, in percent, vitamin-free casein, 18;[2] Salts IV, 4;[3] corn oil, 3;[4] cod liver oil, 1; choline chloride, 0.1, and unmodified wheat starch[5] to 100. Vitamins were supplied in micrograms per gram of diet as follows: thiamine, 2; riboflavin, 3; niacin, 25; calcium pantothenate, 20; *i*-inositol, 100; biotin, 0.1; pteroylglutamic acid, 1; vitamin B_{12}, 0.03; and menadione, 10. Twice weekly each rat received one mg of α-tocopherol acetate dissolved in two drops of corn oil. The basal diet contained 0.07 μg of vitamin B_6 (as pyridoxine hydrochloride) as measured by the method of Atkin et al. ('43).

In the vitamin B_6 bioassay, weanling males were fed the basal diet to which 0.5% of phthalylsulfathiazole was added at the expense of the starch. After three weeks, groups of 6 animals were assigned at random to the various diets. The test diets were fed for 4 weeks and contained either 5 or 10% of ground wheat, 25 or 50% of flour, or 25 or 50% of ground

[2] General Biochemicals.
[3] Phillips and Hart ('35).
[4] Mazola.
[5] Aytex brand, General Mills.

dry bread. These products were added to the phthalylsulfathiazole-containing basal diet at the expense of starch. The standard curve was obtained with 0.0, 0.25, 0.50, 0.75, and 1.50 μg of pyridoxine hydrochloride per gram of diet. One group of animals was fed the basal diet minus phthalylsulfathiazole. All diets were stored at — 20°F. The diets, replenished daily, and distilled water were fed ad libitum. Complete autopsies were performed on three animals at each dosage level. Following paraffin embedding, tissue sections from thyroid, heart, lung, liver, pancreas, adrenals, kidney, gastrointestinal tract, gonads, ureters, urethra and bladder were routinely stained with hematoxylin and eosin. Kidney sections were also stained with Heidenhain's connective tissue stain, Lephene's hemoglobin stain, and by the periodic acid Schiff technique. Both stained and unstained sections were examined with polarized light.

At the conclusion of the bioassay, the 14 animals with the most marked hematuria were selected for further study. All of these animals were on diets containing less than 0.5 μg of vitamin B_6 per gram of diet. Five were given daily intraperitoneal injections of 500 μg of pyridoxine hydrochloride in 0.5 ml of sterile saline. Five animals were given 0.25 ml of salmon egg oil daily by mouth to assure an adequate supply of arachidonic acid as this fatty acid makes up 25% of salmon egg oil (Kyte, '56). The oil was received frozen and sealed under nitrogen. It was thawed slightly, dispensed into small screw-cap vials and stored at — 20°F. until just before feeding. Corn oil was fed as a calorie control. Four rats served as controls. Each animal was continued on the diet it had been fed during the previous period. The course of hematuria and calcium oxalate excretion was observed for a two-week period at the end of which time all 14 animals were autopsied.

Because of the observation (hematuria and oxaluria) a second experiment was designed for further study of this syndrome. Groups of 5 weanling male rats were fed the diets

in table 1 for 9 weeks. Diets 1, 2, 3 and 4 were fed ad libitum. They also were fed using a paired-feeding technique in which the animals were fed as 5 series of one rat per diet. The food intake of each rat in the paired-feeding series on diets 1, 2, 3 and 4 was limited to that of the animal in each group that consumed the least amount. Distilled water was pair-fed by the same technique.

The food dishes and water tubes were removed from the cages from 8:30 A.M. until 4:30 P.M. in order to secure urines relatively free of food particles for microscopic examination. Heavy kraft paper, waxed on both sides, was used under the cages. Urines were inspected daily between 2:00 P.M. and 4:00 P.M. for erythrocytes and for calcium oxalate crystals.

At the end of 9 weeks, the animals were weighed, anesthetized with chloroform and complete autopsies performed. The kidneys were trimmed of fat and connective tissue, emptied of urine if hydronephrotic, and weighed and fixed in 10% formalin and studied histologically.

RESULTS

Bioassay series

Urinary examinations were made daily during the 7th week. They revealed widespread and gross hematuria in groups receiving diets containing 0.5% of phthalylsulfathiazole and less than about 0.35 μg of vitamin B_6 per gram of diet. Microscopic hematuria was occasionally observed in some rats receiving higher levels of the vitamin, including one animal receiving 1.57 μg per gram of diet but it was an uncommon and transient finding. Oxalate excretion appeared to be almost as severe in animals receiving 0.8 μg of the vitamin per gram of diet as it was in animals on lower levels. Calcium oxalate sometimes was observed in urine from only two animals receiving the highest level (1.57 μg per gram) of vitamin B_6.

No significant findings on gross or microscopic examination were noted at post mortem examination of three animals from each dosage level on the standard curve except in the kidneys

TABLE 1

Average food and water intake and weight gain and relative kidney weight at the end of the 9-week period in the second experiment

GROUP	DIET FED	FOOD INTAKE	WATER INTAKE	TOTAL WEIGHT GAIN	KIDNEY WT PER 100 GM BODY WT
		gm/day	*ml/day*	*gm*	*mg*
	Paired feeding (4)[1]				
1	Basal[2]	5.72 ± 0.14[3]	4.51 ± 0.04	33.4 ± 1.2	1150 ± 30
2	Basal + phthalylsulfathiazole[4]	5.72 ± 0.14	4.51 ± 0.04	24.8 ± 5.5	1230 ± 80
3	Basal + phthalylsulfathiazole + pyridoxine[5]	5.72 ± 0.14	4.51 ± 0.04	37.0 ± 2.4	890 ± 50
4	Basal + phthalylsulfathiazole + salmon egg oil[6]	5.72 ± 0.14	4.51 ± 0.04	25.2 ± 4.10	1290 ± 60
	Ad libitum feeding (5)[1]				
1	Basal	6.52 ± 0.49	5.62 ± 0.27	55.2 ± 7.6	1100 ± 50
2	Basal + phthalylsulfathiazole	5.88 ± 0.64	4.87 ± 0.24	48.4 ± 6.7	1130 ± 30
3	Basal + phthalylsulfathiazole + pyridoxine	20.8 ± 1.5	16.0 ± 0.48	280.8 ± 10.1	697 ± 10
4	Basal + phthalylsulfathiazole + salmon egg oil	7.39 ± 0.42	5.24 ± 0.13	50.4 ± 7.6	1170 ± 100
5	Basal + phthalylsulfathiazole + deoxypyridoxine[7]	6.88 ± 0.42	5.51 ± 0.14	50.2 ± 5.9	1180 ± 90
6	Basal + deoxypyridoxine	6.12 ± 0.32	5.83 ± 0.08	60.4 ± 3.4	1080 ± 20
7	Basal + pyridoxine	19.5 ± 1.1	14.8 ± 0.53	260.6 ± 6.1	742 ± 30

[1] Number within parentheses is the number of animals per group.

[2] See text for constituents.

[3] Mean ± standard error of the mean.

[4] Equal to 0.5% of diet.

[5] Five micrograms of pyridoxine hydrochloride added per gram of diet.

[6] Each rat was given 0.1 ml daily for first week and 0.2 ml daily thereafter. Corn oil was fed as a Calorie control.

[7] One microgram of deoxypyridoxine hydrochloride added per gram of diet.

of animals receiving 0.32 μg of vitamin B_6 or less per gram of diet. These animals showed crystalline material in collecting ducts and distal tubules and also occasional deposits high in the calyces (figs. 1, 2). The tubular epithelium was flattened around the crystalline material. The crystals were occasionally surrounded by foreign body giant cells (fig. 1). The crystals were arranged in sheaves and flat rhombic plates and were doubly refractile and stained negatively for calcium, iron and hemoglobin. The doubly refractile quality disappeared two to three minutes after the section was placed in 10% nitric or sulfuric acid but the deposit was not dissolved. Calcium oxalate crystals are reported to disappear on this treatment (Kesten et al., '39). Also, the tissue sections containing crystals gave a positive reaction for sulfathiazole after hydrolysis in 4 N HCl, diazotization and coupling with 1% dimethyl-l-napthylamine.

The architecture of the crystals noted in the deposits is similar to those noted by Gross, Cooper and Scott ('40) in kidneys of rats receiving large doses of phthalylsulfathiazole and their structure and chemical reactions lead to the conclusion that they are acetylsulfathiazole plus, probably, other urinary constituents (Lehr and Antopol, '42).

Investigation of the 14 rats with gross hematuria undertaken at the completion of the bioassay showed that the injection of 500 μg of pyridoxine hydrochloride per rat per day had no effect on the hematuria but did greatly decrease urinary excretion of calcium oxalate crystals at the time of the first observation (three hours after injection). After 16 hours, no calcium oxalate crystals were observed in the urine. The oral administration of salmon egg oil to the vitamin B_6-deficient animals for a two-week period was without effect on either calcium oxalate excretion or hematuria. The urinary findings remained unchanged in the 4 untreated control rats. Weight gains averaged 40 gm per rat per week for the injected group, and only 10 gm per rat per week for the salmon egg oil and control groups.

At autopsy, gross kidney lesions were only observed in the salmon egg oil group where three of the 5 animals showed varying degrees of hydronephrosis involving one or both kidneys. The pelves, ureters and bladders contained urine.

Microscopic examination of the animals with grossly visible hydronephrosis (figs. 2, 3) showed areas of acute pyelonephritis with hemorrhage into calyces. Dilatation of individual calyces with some hemorrhage was noted in 4 other animals all of which showed crystalline material in the collecting ducts or attached to the pelvis, usually high in a calyx. Dilated tubules were common in the cortex of all animals. No red cell casts were noted in any proximal tubules, though red cells were present enmeshed in casts in the collecting ducts of one animal. The crystalline material was identical in character to that described in the animals on the standard curve.

Second series

Growth data. Weight gain and food and water intakes for each group of animals are given in table 1. One animal in one pair-fed series of group 4 died on the 56th day. Therefore, data on this series are not included.

Under the paired-feeding conditions, pyridoxine did not significantly influence weight gain (group 3 versus 2, $P > 0.05$). Analysis of variance showed that phthalylsulfathiazole, salmon egg oil, and deoxypyridoxine singly or in combination did not affect weight gain ($P > 0.05$). The controlled feeding technique resulted in smaller weight gains than were obtained under ad libitum feeding conditions ($P < 0.05$).

The beneficial effect of pyridoxine under ad libitum feeding conditions is obvious. Analysis of variance showed that food and water intakes were unaffected by phthalylsulfathiazole, salmon egg oil, or deoxypyridoxine feeding. Statistical analysis of intakes obtained by both methods of feeding showed the reductions in food intakes caused by paired-feeding to be significant in case of groups 1 and 4 ($P < 0.01$). Paired-feeding restricted the water intake of group 4 only ($P < 0.01$).

Calcium oxalate crystal excretion. The times required for the first appearance of calcium oxalate crystals in the urine after the animals were placed on the vitamin B_6-deficient diets, are given in table 2. No animal receiving the vitamin supplement excreted calcium oxalate crystals. Oxalate excretion was observed within three days of feeding the vitamin B_6-deficient diets with all deficient animals excreting calcium oxalate by the 15th day.

Hematuria. The time that erythrocytes first appeared in the urine is indicated in table 2. Microscopic hematuria appeared in pair-fed animals receiving diets 2 and 4 and not in diets 1 and 3. Diets 2 and 4 are vitamin B_6-deficient diets containing phthalylsulfathiazole with and without salmon egg oil. Similar findings were noted in the ad libitum-fed animals where groups 2 and 4 both showed microscopic hematuria about the same time as the pair-fed groups. In addition, animals on diet 5 which contained deoxypyridoxine and phthalylsulfathiazole also showed microscopic hematuria. No hematuria was observed on the diet containing deoxypyridoxine without phthalylsulfathiazole. Also, no hematuria was noted in animals in group 7 where the basal diet was supplemented with pyridoxine hydrochloride. The hematuria was gross at some time in 17 and a daily occurrence in 12 of the 25 animals in pair-fed groups 2 and 4 and ad libitum-fed groups 2, 4 and 5. No hematuria was observed unless phthalylsulfathiazole was fed in the vitamin B_6-deficient diet, except for one animal noted in group 3 fed ad libitum. In the most severely affected animals the intensity of hematuria often varied over a period of hours. It was noted that when hematuria was most severe, excretion of calcium oxalate crystals was greatly diminished or ceased entirely.

Autopsy findings. The genitourinary tracts were examined and showed findings similar to those noted in the bioassay series. No abnormalities were observed in the kidneys of animals fed diets containing 5 μg of added pyridoxine hydrochloride per gram of diet with phthalylsulfathiazole and the

kidneys were normal in animals with zero levels of added pyridoxine when phthalylsulfathiazole was omitted from the diet. Animals with zero levels of added pyridoxine plus phthalylsulfathiazole showed gross hematuria and at autopsy the pelves and ducts of Bellini contained doubly refractile

TABLE 2

Time of appearance of calcium oxalate crystals in the urine and hematuria given in days in the second experiment

GROUP	CALCIUM OXALATE CRYSTALS OBSERVED		HEMATURIA OBSERVED		CONSISTENT GROSS HEMATURIA
	First	In all rats	First	In all rats	
					% of animals
		Paired feeding			
1	7	15	none	none	—
2	3	13	17	48 [1]	80
3	none	none	none	none	—
4	3	10	12	35	60
		Ad libitum feeding			
1	7	13	none	none	—
2	4	12	25	43	60 [2]
3	none	none	37 [3]	—	—
4	4	9	13	32	80 [4]
5	7	10	15	52	60
6	5	10	none	none	—
7	none	none	none	none	—

[1] Only 4 of the 5 animals in this group showed hematuria throughout the entire course of the experiment.

[2] Showed gross hematuria but was more intermittent in character than group 2 of the pair-fed animals.

[3] One animal showed sporadic hematuria (never gross) from this date to the end of the experiment.

[4] Two animals with intermittent and two animals with consistent gross hematuria.

crystalline material (fig. 4) admixed with eosinophilic debris and erythrocytes. Some dilated tubules, particularly collecting ducts and Henle's loops tubules, were found in almost all these kidneys.

The ureters were dissected into the bladder in all instances. Three animals with zero levels of added pyridoxine receiving

phthalylsulfathiazole showed ureteral calculi. The kidneys of these animals were hydronephrotic as were kidneys of two other animals in this group. The calculi were ovoid and were estimated to have distended the ureters to a diameter three to 4 times normal. They could not be moved to the bladder by gentle probing. Calculus analysis was not performed.

Kidney weights expressed as milligrams per 100 gm of body weight are given in table 1. Analysis of variance showed that the kidneys of the vitamin B_6-deficient animals were significantly heavier relative to body weight than those of the control animals ($P < 0.05$). Tissue water determinations were not performed so edema cannot be ruled out as a cause of the weight increase. Phthalylsulfathiazole, salmon egg oil, deoxypyridoxine, or the method of feeding was without effect on kidney weight ($P > 0.05$).

DISCUSSION

Paired-feeding of food and water in experiment 2 showed that vitamin B_6 deficiency predisposed the animals to calcium oxalate excretion whether or not 0.5% of phthalylsulfathiazole was included in the diet. Pyridoxine levels 50% higher than the 1 μg per gram of diet considered adequate for growth did not completely prevent oxalate excretion in all animals.

The appearance of calcium oxalate crystals in the urine only three days after animals are placed on a vitamin B_6-deficient diet indicates that urinary calcium oxalate may offer a simple index of suboptimal vitamin B_6 intake. Its origin may represent faulty glycine metabolism. Nakada and Weinhouse ('53) have shown *in vitro* conversion of glycine to oxalate by rat liver homogenates in the presence of high levels of glyoxylate, the deamination product of glycine.

The mechanism for the normal decarboxylation of glyoxylate may be very sensitive to a deficiency of vitamin B_6, resulting in an accumulation of sufficient glyoxylate to permit oxalate formation. A decreased rate of transamination toward glycine could also permit accumulation of excessive glyoxylate

with subsequent oxalate formation. The authors have evidence that a failure of one of these mechanisms may explain the calcium oxalate excretion because the feeding by stomach tube of 100 mg of glycine per 100 gm of body weight to a fasted vitamin B_6-deficient rat markedly increased calcium oxalate excretion. The administration of glucose, sucrose or corn oil had no such effect. Sodium benzoate administered to a vitamin B_6-deficient rat eliminated calcium oxalate excretion, presumably by combining with glycine to form hippuric acid (Archer et al., '57).

Hematuria was found only in animals that were vitamin B_6-deficient and receiving phthalylsulfathiazole. However, the hematuria was probably not due to the presence of a more severe vitamin B_6 deficiency in rats fed phthalylsulfathiazole because statistical analysis of the weight data showed the vitamin B_6 deficiency was no more severe in this group than in a deficient group without the sulfa drug. The lack of beneficial effects of salmon egg oil (source of arachidonic acid) on the course of hematuria and development of kidney abnormalities indicates that the syndrome reported here is not similar to that reported in rats with essential fatty acid deficiency (Burr and Burr, '29, '30).

The autopsies showed the source of the hemorrhage to be around foci of crystal deposition in the urinary pelvis with or without acute pyelonephritis and partial hydronephrosis. The crystalline material is believed to be acetylsulfathiazole as it shows many of the characteristics described for this material (Lehr and Antopol, '42; Hawking and Lawrence, '51), and the doubly refractile crystals in the tissues and the hematuria were found only in rats fed phthalylsulfathiazole.

Several hypotheses can be suggested to explain why vitamin B_6-deficient rats fed phthalylsulfathiazole develop crystal deposits and hematuria and rats fed normal pyridoxine levels and receiving phthalylsulfathiazole do not. The actual etiology is not known. One hypothesis is that sulfathiazole and acetylsulfathiazole are precipitated in the acid urine of vita-

min B_6-deficient rats fed this drug. Both free and acetylsulfathiazole are known to be less soluble in acid urine (Hawking and Lawrence, '51). In addition, vitamin B_6 deficiency might be associated with increased acetylation of sulfathiazole with consequent increased urinary excretion of this much less soluble material. It is possible that the crystal deposits are a combination of oxalate and acetylsulfathiazole and hence only occur in the deficient animals which have oxaluria.

SUMMARY

Weanling male albino rats were fed a vitamin B_6-deficient diet containing 0.5% of phthalylsulfathiazole for periods up to 9 weeks. Profuse urinary excretion of calcium oxalate crystals occurred in all animals, and gross hematuria was common. Many of the rats receiving less than about 0.35 μg of the vitamin per gram of diet showed some degree of hydronephrosis and several had ureteral calculi. Histological studies showed the hematuria originated around foci of crystal deposition in the pelvis of the kidney with or without an associated hydronephrosis.

Calcium oxalate excretion, but no crystal deposition in the kidney, kidney damage or hematuria occurred when the deficient diet minus phthalylsulfathiazole was fed. A vitamin B_6 level of 1.57 μg per gram of diet, which is about 50% higher than the generally accepted level required for growth, did not prevent the excretion of calcium oxalate crystals in some rats.

Supplementation with 5 μg of pyridoxine hydrochloride per gram of diet prevented urinary excretion of calcium oxalate crystals and was effective in preventing kidney damage and hematuria when phthalylsulfathiazole was fed.

The crystal deposits in the kidney contained sulfathiazole and the results of the experiments cited above indicate that the excretion of this compound in vitamin B_6-deficient rats was directly associated with renal damage and hematuria.

ACKNOWLEDGMENTS

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PLATE 1

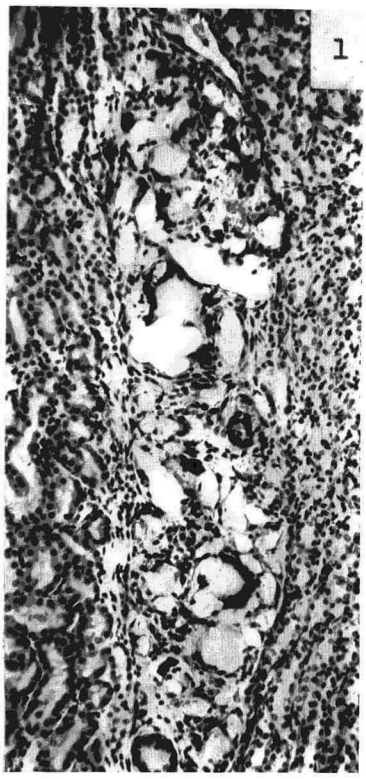
EXPLANATION OF FIGURES

1 Section taken high through a calyx of rat no. 15 which was in the bioassay series on the basal diet plus phthalylsulfathiazole and receiving 0.32 μg of vitamin B_6 per gram of diet. The animal had persistent oxaluria and gross hematuria. Note the foreign body type giant cell reaction around acicular spaces. Some remnants of doubly refractile material were present when this area was examined with polarized light. Hematoxylin and eosin stain, $\times$ 130.

2 Section through renal pelvis in an area of acute pyelonephritis with crystal deposition and hemorrhage in rat no. 6. This animal had oxaluria and hematuria and received salmon egg oil supplement. This photograph was taken under partially crossed Nicol prisms to show the doubly refractile crystals. Hematoxylin and eosin stain, $\times$ 105.

3 Low power photomicrograph of same animal shown in figure 2 to demonstrate hemorrhage into pelvis of hydronephrotic kidney of this rat. Hematoxylin and eosin stain, $\times$ 42.

4 Relatively high power photomicrograph to show crystal detail in a distal tubular deposit under almost completely crossed Nicol prisms. Hematoxylin and eosin stain, $\times$ 240.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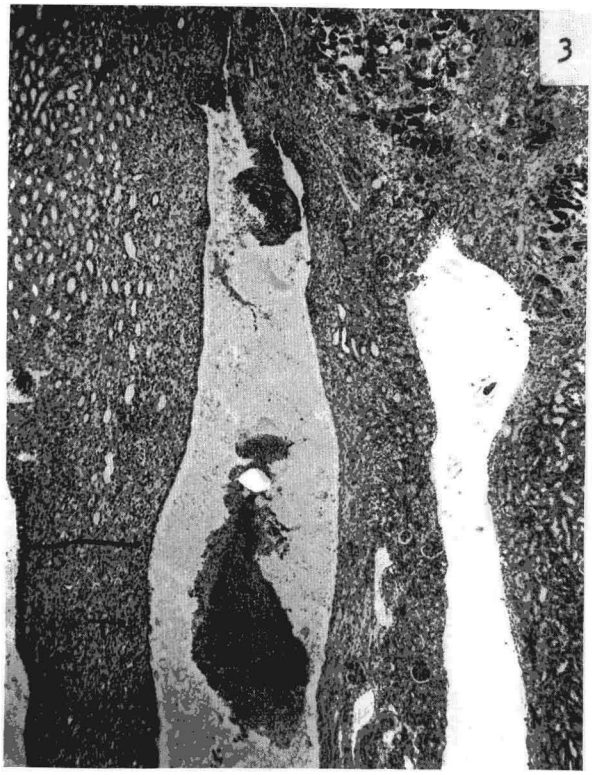
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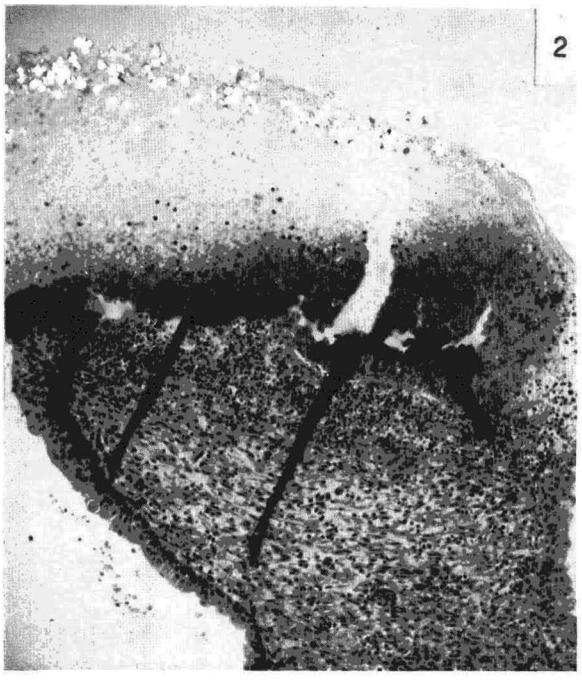
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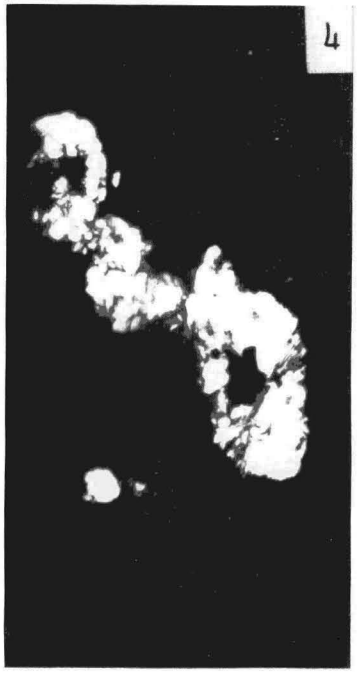
4

STUDIES ON THE RELATION OF DAIRY PRODUCTS TO DENTAL CARIES IN CARIES-SUSCEPTIBLE RATS [1]

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The development of satisfactory experimental caries assay procedures and the availability of dependable strains of caries-susceptible rats make possible careful studies in which the influence of supplements of human foods to a cariogenic diet can be tested either during the post-eruptive period or during the developmental period. The present study was concerned with the supplementation of a cariogenic ration either with individual dairy products or with combinations of dairy products. These supplements were provided either during the developmental period but not in the post-eruptive period, or during the post-eruptive period but not in the developmental period, or throughout both the developmental and post-eruptive periods. The levels of supplementation were chosen to fall within likely ranges of human consumption for the age periods under investigation. The supplementation with dairy products was in every test at the proportionate expense of all ingredients in the cariogenic ration rather than by the replacement of a single dietary component which in many cases would introduce an immediate bias into the trial.

Two experiments were conducted: one in which the influences of milk, chocolate drink and chocolate milk were compared; the

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other in which a mixture of milk, ice cream and cheese and a mixture of chocolate milk, ice cream and cheese were compared. Comparable experiments could be conducted with practically any other foodstuff.

EXPERIMENTAL

General experimental design

Parental generation. Two generations of rats were represented in each of the two experiments: the parental and the filial generation. The rats in the parental generation were females that were carefully selected at weaning from the Harvard caries-susceptible colony to insure a high degree of uniformity in caries susceptibility. The females in this generation were subdivided equitably on the basis of littermate distribution into the appropriate control (unsupplemented) and experimental (supplemented) groups. The primary function of the experimental females in the parental generation was to produce offspring whose development during pregnancy and lactation took place during the consumption of a dietary supplement by their mothers. The caries incidences of these offspring were compared with those of the offspring from control females as a measure of whether or not the consumption of the dietary supplements had influenced the inherent caries-susceptibility of the teeth.

In addition, the females in the parental generation may serve one of two additional purposes. If the dietary supplement was begun at weaning, these females could be used in a preliminary test of the post-eruptive influence of the supplement. In addition, this prolonged period of supplementation would provide the maximum opportunity for the supplement to make its influence felt upon the mothers and their offspring. This procedure was followed in experiment one, where the supplements of milk, chocolate drink and chocolate milk were begun immediately after weaning. On the other hand, the supplement could be withheld until a litter of offspring had been born while the females were still on the unsupplemented

cariogenic dietary regimen. This litter would serve as a caries-susceptibility control for all later litters by the same parents. Then the supplement could be begun, maintained through an equilibrium period and throughout the later reproductive periods. This procedure was followed in the second experiment.

The first procedure has the advantage of providing a test of the purely post-eruptive influence of the supplements but not under ideal conditions because the length of time that these females were on experiment was in excess of the most desirable caries assay period. In other words, the experimental duration was sufficiently long for many of the rats in the control group to develop the maximum amount of tooth decay long before the termination of the experiment; then the rats in the experimental groups would have time for their caries scores to increase toward the maximum sufficiently to obscure any earlier experimental difference.

Filial generation. As soon as litters of these females were weaned, the weanlings were divided into two or more subgroups. The rats in the first subgroup in each case were maintained as controls on the unsupplemented cariogenic ration. These animals served as the test subjects for the determination of whether or not the dietary supplement during pregnancy and lactation had influenced the genetically expected level of caries susceptibility. The remaining subgroup or subgroups were maintained with a dietary supplement to determine whether that supplement was capable of a post-eruptive influence on the dental caries incidence. Where the females' diet had been unsupplemented, the supplemented diets for the subgroups represented a test of whether the supplement had any independent post-eruptive influence. Where the females' diet was supplemented and the diets of the offspring were unsupplemented the test indicated whether the supplement during pregnancy and lactation had a developmental influence on the caries-susceptibility of the offspring. Where both the females' diet and the offsprings' diet were supplemented, an index is

obtained of whether there is an additive effect by reason of both the developmental and post-eruptive supplementation.

Design of experiment 1

The details of the first experiment are listed in the first 7 columns of table 1. Thirty-eight weanling female rats were divided into 4 groups. The rats in group 1 received cariogenic diet 900 + 15% of Cellu flour throughout the entire experimental period without supplementation. With two exceptions, this ration is identical to our customary cariogenic ration 700 + 15% of Cellu flour (Resnick, Willett and Shaw, '58). The 40 gm of desiccated liver contained in each 1190 gm of ration 700 + 15% of Cellu flour were omitted. Ration 900 + 15% of Cellu flour contained supplements of 20 mg biotin, 200 mg of folic acid and 0.1 mg of crystalline vitamin B_{12} in each 1190 gm of ration, whereas these supplements were not added to ration 700 + 15% of Cellu flour because of its liver content. The rats in groups 2, 3 and 4 received supplements of milk, chocolate drink and chocolate milk, respectively, in adequate amounts to provide 30, 30 and 35%, respectively, of the daily caloric intake. After 24 weeks on these regimens, the females were bred with suitable caries-susceptible males from the stock colony, during which time the same dairy supplements were maintained.

When each litter was weaned, the offspring were divided into two subgroups, A and B. One half of each litter (A) was maintained for 100 days on ration 900 + 15% of Cellu flour. The other subgroup (B) from each litter received a dairy product exclusively for the first 42 days after weaning, at which time they were transferred to a regimen consisting of 33% of the Calories from a supplement of the same dairy product and 67% of the Calories from ration 900 + 15% of Cellu flour for the remaining 58 days. In control group 1, subgroup B was given milk as its supplement, whereas in experimental groups 2, 3 and 4, the subgroup B in each litter was given the same dairy supplement as their mothers had received, milk, chocolate drink and chocolate milk, respectively.

TABLE 1

The influence of milk, chocolate drink and chocolate milk supplements to a cariogenic dietary regimen on the incidence of dental caries

GROUP AND SUBGROUP	LEVEL OF DAIRY PRODUCT IN MOTHER'S DIET	POST-WEANING DAIRY PRODUCT SUPPLEMENT	LEVEL OF DAIRY PRODUCT IN DIET AFTER WEANING		NUMBER OF RATS	POST-WEANING EXPERIMENTAL DURATION	NUMBER OF CARIOUS MOLARS			NUMBER OF CARIOUS LESIONS			EXTENT OF CARIOUS LESIONS		
			21–63 days of age	After 63 days of age			Average	SEM [1]	CR [2]	Average	SEM [1]	CR [2]	Average	SEM [1]	CR [2]
	% day's Calories		*% day's Calories*	*% day's Calories*		*days*									
Maternal generation															
1	0	None	0	0	12	409	10.2	0.5	3.8	19.8	1.0	3.6	83.5+	6.5+	3.6
2	0	Milk	30	30	9	426	5.4	1.2	3.1	10.8	2.3	3.2	41.4+	9.9+	2.8
3	0	Chocolate drink	30	30	8	420	6.6	1.1	3.2	12.2	2.2	3.6	51.9+	9.3+	3.9
4	0	Chocolate milk	35	35	9	416	6.1	1.2		11.8	1.9		45.7+	7.2+	
Filial generation															
1A	0	None	0	0	36	100	10.4	0.4	21.5	17.4	0.9	14.3	52.1+	3.8+	12.7
1B	0	Milk	100	33	35	100	1.6	0.1	11.7	2.0	0.6	10.6	2.7+	0.8+	10.7
2A	33	None	0	0	30	100	10.1	0.5	2.2	17.0	1.2	2.1	52.5+	4.5+	2.2
2B	33	Milk	100	33	23	100	1.8	0.5	4.4	2.2	0.7	4.8	3.0+	1.1+	5.3
3A	33	None	0	0	22	100	9.9	0.5	2.5	16.7	1.1	2.0	50.6+	5.0+	2.4
3B	33	Chocolate drink	100	33	14	100	4.2	1.2	5.0	6.2	1.9	5.2	13.6+	4.9+	5.1
4A	33	None	0	0	23	100	8.7	0.8		13.7	1.4		40.3+	5.5+	
4B	33	Chocolate milk	100	33	28	100	3.4	0.7		4.5	1.1		9.2+	2.6+	

[1] SEM = Standard error of mean.

[2] CR = Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when from 2.0 to 2.9, moderately significant; when 3.0 or higher, highly significant.

Design of experiment 2

The details of the second experiment are listed in the first 7 vertical columns of table 2. Forty-five females were taken from the stock colony and divided into three groups, all of which were maintained on ration 700 + 15% of Cellu flour until their first litter was weaned. Immediately thereafter the females in the second group were begun on a regimen where 45% of the Calories was supplied by the plain dairy supplement containing milk, vanilla ice cream and cheddar cheese in the proportions described below, and 55% of the Calories by ration 700 + 15% of Cellu flour. The females in the third group were on the same type of regimen except that 45% of Calories was supplied by the chocolate dairy supplement containing chocolate milk, vanilla ice cream and cheddar cheese in the proportions described below. The females in the first group were continued on ration 700 + 15% of Cellu flour throughout. At an appropriate period of three to 4 weeks after their first litters were weaned, the females were bred again and were allowed to complete as many reproductive cycles as possible.

The first series of litters from all three groups of females that were born and reared while the mothers were consuming ration 700 + 15% of Cellu flour were each divided into three subgroups, A, B and C. The rats in subgroup A from each group of female rats were transferred to ration 700 + 15% of Cellu flour without supplementation and maintained thereon until sacrificed 85 days after weaning. The rats in subgroup B from each litter were fed either the plain or the chocolate dairy supplement exclusively for the first 21 experimental days and then transferred to a regimen where 33% of the Calories was provided by one of the dairy supplements and 67% by ration 700 + 15% of Cellu flour. These rats also were sacrificed 85 days after weaning. The rats in subgroup C from each litter were treated identically except that they were continued on this regimen for 85 days, i.e. a total of 106 days after weaning. The latter time arrangement was provided so that

the rats in subgroup C would have had the same length of exposure to cariogenic ration 700 + 15% of Cellu flour as the rats in subgroup A. The plain dairy supplement was provided to the rats in subgroups B and C of the litters from the first and second groups of females. The chocolate dairy supplement was provided to the rats in subgroups B and C of the litters from the third group of females.

After the dairy supplements were begun for the females in groups 2 and 3 of the parental generation, that is, after their control series of litters had been weaned, two additional series of litters were raised. The rats in each litter of the second series of litters were subdivided into three subgroups, D, E and F, which were provided the same diets as the rats in subgroups A, B and C, respectively, of the control series of litters. The only difference between these two series was the absence of dairy supplements in the maternal diet during the production of the first series and the presence of dairy supplements in the maternal diet during the production of the second series of litters. The rats in the third series of litters were subdivided into two subgroups, G and H, which were comparable to subgroups B and C, respectively, and to subgroups E and F, respectively, except that lower levels of the dairy supplements were fed post-eruptively. During the first 21 days after weaning, 45% of the Caloric requirements was provided by the dairy supplements instead of 100%, and in the periods after 42 days of age 25% of the Caloric requirements was provided by the dairy supplements instead of 33%.

Throughout both experiments, the rats were housed in individual wire-bottom cages for all periods of the experiments except during the reproductive cycles when they were kept in cages with soft pine shavings as bedding. Tap water was provided ad libitum. At the termination of the appropriate periods, the rats were sacrificed and the heads stored in 95% alcohol for 48 hours or more. The incidence of dental caries was determined by the method described by Shaw, Schweigert, McIntire, Elvehjem and Phillips ('44).

TABLE 2

The influence on dental caries incidence of dairy product supplements containing either milk, vanilla ice cream and cheddar cheese, or chocolate milk, vanilla ice cream and cheddar cheese to a cariogenic dietary regimen

GROUP AND SUBGROUP	LEVEL OF DAIRY PRODUCT SUPPLEMENTS IN MOTHER'S DIET	POST-WEANING DAIRY PRODUCT SUPPLEMENT	LEVEL OF DAIRY PRODUCT SUPPLEMENT IN DIETS AFTER WEANING		NUMBER OF RATS	POST-WEANING EXPERIMENTAL DURATION	NUMBER OF CARIOUS MOLARS			NUMBER OF CARIOUS LESIONS			EXTENT OF CARIOUS LESIONS		
			21–42 days of age	After 42 days of age			Average	SEM [1]	CR [2]	Average	SEM [1]	CR [2]	Average	SEM [1]	CR [2]
	% day's Calories		*% day's Calories*	*% day's Calories*		*days*									
Maternal generation															
1	0	None	0	0	12	333	10.2	0.6	1.4	21.2	1.0	1.5	94.0+	5.1+	1.7
2	0	Plain	0	45 [3]	15	399	8.8	0.7	1.8	18.9	1.2	1.5	81.9+	5.2+	1.7
3	0	Chocolate	0	45 [3]	18	354	8.7	0.6		19.1	1.0		82.9+	3.8+	
Filial generation															
1A	0	None	0	0	34	85	8.0	0.5	11.8	13.1	1.1	10.8	43.6+	3.7+	10.9
1B	0	Plain	100	33	15	85	0.7	0.3	11.3	0.9	0.4	10.5	1.9+	0.9+	9.8
1C	0	Plain	100	33	17	106	0.9	0.3		1.1	0.4		3.4+	1.7+	

2A	0	None	0	0	28	85	8.6	0.5	12.6	15.0	1.0	11.8	52.4+	4.1+	11.3
2B	0	Plain	100	33	13	85	0.8	0.4	14.1	1.2	0.7	14.4	3.2+	1.6+	12.1
2C	0	Plain	100	33	14	106	0.7	0.3	0.7	0.7	0.3	1.1	2.3+	0.9+	0.5
2D	45	None	0	0	32	85	8.1	0.5	13.9	13.5	0.9	13.7	49.8+	3.3+	13.9
2E	45	Plain	100	33	16	85	0.6	0.3	16.1	0.6	0.3	14.6	1.9+	1.0+	14.9
2F	45	Plain	100	33	16	106	0.2	0.1	4.6	0.2	0.1	4.9	0.5+	0.3+	4.2
2G	45	Plain	45	25	8	85	3.9	0.9	6.9	6.2	1.5	5.8	22.6+	5.6+	4.5
2H	45	Plain	45	25	8	106	3.1	0.7		5.0	1.4		18.8+	6.1+	
3A	0	None	0	0	34	85	6.3	0.5	8.1	10.1	1.0	7.8	34.6+	3.8+	6.9
3B	0	Chocolate	100	33	17	85	1.2	0.4	4.1	1.5	0.5	3.6	4.6+	2.1+	2.8
3C	0	Chocolate	100	33	17	106	2.5	0.8	0.3	3.9	1.4	0.6	14.5+	6.0+	0.1
3D	45	None	0	0	35	85	6.5	0.5	7.1	10.9	1.0	6.8	38.4+	4.3+	5.6
3E	45	Chocolate	100	33	17	85	1.6	0.5	4.9	2.3	0.8	5.0	8.4+	3.3+	4.2
3F	45	Chocolate	100	33	16	106	2.6	0.6	5.6	3.9	1.0	5.8	14.4+	3.8+	5.5
3G	45	Chocolate	45	25	21	85	2.4	0.5	6.7	2.9	0.2	6.6	9.4+	2.3+	5.8
3H	45	Chocolate	45	25	17	106	1.6	0.5		2.1	0.7		7.6+	2.7+	

[1] SEM = Standard error of mean.
[2] CR = Critical ratio.
[3] Supplement begun after first litter weaned.

Preparation and use of supplements

In the first experiment, the three supplements, milk, chocolate milk, and chocolate drink, were prepared from the same vat of raw milk. The milk supplement was regular homogenized milk prepared as if for the retail trade. The chocolate milk was prepared by the addition of a chocolate syrup without previous alteration of the fat content. The chocolate drink was prepared by the use of the same chocolate syrup, but with a reduction of the fat content of the product to approximately 2% as is customary in Massachusetts in the preparation of chocolate drink for retail sale. Ample amounts of these preparations for the entire experimental period were prepared before the beginning of the experiment, frozen immediately in quart cartons, and stored to minimize as much as possible any variation in the composition of these supplements during the long-term experiment.

Small amounts of each of these preparations were withdrawn from the storage plant as needed and kept in a household freezer in the laboratory. Each afternoon an adequate amount was taken from the freezer and allowed to thaw in a refrigerator in preparation for the next day's feedings. On the basis of the analyses [2] of the preparations, appropriate amounts of each supplement were provided to the various groups of rats as outlined in the first 7 vertical columns in table 1.

In the second experiment, two supplements were tested, each of which contained three dairy products. The plain dairy supplement contained milk, vanilla ice cream, and medium cure cheddar cheese. The chocolate dairy supplement contained chocolate milk, vanilla ice cream, and medium cure cheddar cheese. As in the first experiment, the milk and chocolate milk were prepared by comparable procedures from the same vat of raw milk. Ample amounts for the entire

[2] Regular homogenized milk contained 3.75% fat and 8.59% non-fat solids; chocolate milk contained 3.09% fat and 19.66% non-fat solids; and chocolate drink contained 1.98% fat and 14.84% non-fat solids.

experimental period were frozen and stored. The ice cream was taken from one batch in the ice cream plant and set aside under appropriate refrigeration until withdrawn as needed. The medium cure cheddar cheese was provided in the form of 40-pound blocks from the same batch that were frozen until needed.

To avoid any problems of preferential selection of the three portions of the two supplements by the rats, mixtures of the ingredients were prepared. The cheddar cheese was ground into fine particles. This subdivision was done in a feed mill operating at approximately —10°C. In advance of the grinding, the frozen cheese had been cut into small chunks not exceeding one-half inch in any direction. These chunks were thoroughly mixed with similar sized pieces of dry ice. When the cheese and dry ice were fed slowly into a pre-cooled mill, the cheese was ground to an ideal particle size. On the basis of the analyses [3] of the ingredients, appropriate amounts of cheese, ice cream, and partially thawed milk or partially thawed chocolate milk were thoroughly blended in a Hobart food mixer. The percentages of Calories provided in the plain and chocolate dairy supplement were: milk or chocolate milk 66.3, ice cream 23.2, and cheese 10.5. The mixtures of dairy products were held at a sufficiently low temperature during mixing to keep the entire mass at a heavy plastic consistency. The supplements were then packaged in small cartons and returned to the freezer. Ample amounts of each of the plain dairy supplement and chocolate dairy supplement were prepared at one time to last for approximately 6 weeks. The consistency of these supplements at freezer temperatures approximated that of relatively soft ice cream which enabled easy measurement and use for the supplementation of the appropriate animals without preliminary thawing. Appropri-

[3] Regular homogenized milk contained 3.57% fat and 8.46% non-fat solids; chocolate milk contained 2.94% fat and 19.34% non-fat solids; ice cream contained 11.52% fat, 16.1% sugar and 10.0% non-fat milk solids; cheddar cheese contained 22.0% protein, 35.1% fat, 3.54% ash, 35.45% moisture, 0.60% calcium, 0.50% phosphorus, 1.48% sodium chloride and no measurable lactose.

ate amounts of these supplements were provided to the various groups of rats as outlined in the first 7 vertical columns in table 2.

RESULTS

There were no clear distinguishing physical characteristics between the control rats and the comparable experimental rats that received the dairy product supplements in both experiments 1 and 2 other than slightly slower rates of growth among the supplemented rats in the early experimental weeks. Before the end of the respective experimental periods, the supplemented rats and the control rats had attained comparable average body weights. The following characteristics were the same for the control and supplemented rats: fertility of the females in the maternal generation, litter size, weaning weight and viability of the offspring, general health and well-being of the representatives of both the maternal and filial generations, and bone size, composition and structure.

The influences of milk, chocolate drink and chocolate milk on dental caries incidence as compared in experiment 1 are tabulated in table 1. In the maternal generation where the females in experimental groups 2, 3 and 4 were fed dairy product supplements from weaning until the time that they were sacrificed, major reductions in dental caries incidence occurred as a result of this post-eruptive feeding of the supplements. When these reductions were considered in terms of any one of the three criteria, the number of carious molars, the number of carious lesions, or the extent of carious lesions, the influence of each of the three supplements, milk, chocolate drink and chocolate milk at levels of 30, 30 and 35% of the day's Caloric intake, respectively, was observed to be highly significant. Although there were no significant differences between the influence of the three supplements, the milk supplement tended to be the most effective, the chocolate drink supplement least effective, with the chocolate milk supplement intermediate. Because of the small differences between the averages for the three supplements, very large groups of rats would be

necessary to demonstrate whether this trend was statistically significant.

In the filial generation, the feeding of any one of these three dairy products to the weanling rats in subgroups 1B, 2B, 3B and 4B under the conditions of this experiment resulted in highly significant reductions in dental caries as measured by any one of the three criteria for the evaluation of dental caries and compared to their control littermates in subgroups 1A, 2A, 3A and 4A, respectively. In many of the rats in subgroups 1B, 2B, 3B and 4B, dental caries was completely prevented, whereas practically every rat in subgroups 1A, 2A, 3A and 4A had extensive carious lesions. This demonstration of a post-eruptive influence of dairy supplements occurred regardless of whether or not the females in the maternal generation had received a dairy supplement. The supplements used in this section were reasonably high. During the first 42 days post-weaning, the dairy product supplied all of the Caloric requirements; thereafter, only 33% of the Caloric requirements was provided by the supplement. In other words, the rats in subgroups B had had no exposure to a cariogenic diet until 63 days of age and then only for 58 days. As in the maternal generation, there was a tendency for the milk supplement to be most effective and the chocolate drink supplement to be least effective, with the chocolate milk supplement intermediate. However, in these comparisons, unlike the maternal generation, the differences between the supplements were of borderline statistical significance.

The feeding of the dairy product supplements during pregnancy and lactation in order to be available during the development of the teeth of the offspring did not influence the caries susceptibility of the teeth. When the dental caries incidences of the rats in subgroups 2A, 3A and 4A, whose mothers received a supplement throughout reproduction, are compared with the dental caries incidence of the rats in subgroup 1A, whose mothers did not receive a supplement, it is evident that no statistically significant reductions in dental caries incidence were produced.

The influences of the plain dairy supplement and the chocolate dairy supplement on dental caries incidence as tested in experiment 2 are presented in table 2. In the maternal generation where the females in experimental groups 2 and 3 were fed one of the above supplements after their first litter was weaned until the end of the experimental period, modest reductions in dental caries incidence were observed. Although these reductions were not of statistical significance, the trend toward reduced dental caries incidences in the supplemented female rats is noteworthy largely because of the late period in their lives at which the supplement was begun. The two supplements had almost identical effectiveness within our ability to evaluate the extent of the carious process.

In the filial generation, the dental caries incidences for the rats in subgroups 1A, 2A and 3A represent the control measure of the caries-susceptibility of the three populations of rats used in the three groups of the experiment. The rats in subgroup 2A tended to have the highest caries-susceptibility, those in subgroup 3A the lowest, with those in subgroup 1A intermediate, although none of the differences were of major importance. It is against these values that the values for subgroups B through H should be compared in each group because the rats in subgroup A did not receive any supplement posteruptively, nor did their mothers during this first reproductive cycle.

The feeding to the rats in subgroups B and E throughout groups 1, 2 and 3 of either one of the two dairy product supplements at a level of 100% of the Caloric needs during the first 21 days after weaning and at a level of 33% for the remaining 64 days of the experiment caused major reductions in dental caries incidence. In many of the rats in these subgroups, no carious lesions developed, whereas practically every rat in subgroups A had extensive carious lesions. In subgroups C and F where the same levels of supplementation were provided but where the experimental period was extended for an additional 21 days, major reductions in dental caries

incidence were again produced, with numerous caries-free rats in each subgroup. Subgroups C and F in this experiment have an important role in the experimental design because the rats in these subgroups were exposed to the caries-producing diet for a total of 85 days, the same length as the control rats in subgroups A and D. In contrast, the rats in subgroups B and E had only 64 days exposure to the cariogenic diet. In the case of the plain dairy supplement in group 2, the caries incidences of the rats in subgroups 2C and 2F had not increased above the caries incidences of the rats in subgroups 2B and 2E despite the 21-day longer exposure to the cariogenic diet. In the case of the chocolate dairy supplement, modest increases in the dental caries incidences were observed for the rats in subgroups 3C and 3F beyond those observed in 3B and 3E. The comparison of the caries incidences for the rats in groups 3B, 3C, 3E and 3F where the chocolate dairy supplement was fed with those for the rats in groups 2B, 2C, 2E and 2F where the plain dairy supplement was fed indicate a trend toward a higher caries incidence for the rats in group 3 than group 2. This trend may be of importance in view of the tendency for the rats in group 3 to have a lower genetic tendency toward caries-susceptibility than those in group 2 as judged by the comparison of the caries values for subgroups 3A and 3D versus subgroups 2A and 2D. However, on the strict basis of a statistical comparison, the higher caries incidences of the rats supplemented with the chocolate dairy supplement were not significantly greater than those of comparable rats that received the plain dairy supplement.

Where the lower levels of the plain and chocolate dairy supplements were fed post-eruptively to the rats in subgroups G and H, large reductions in dental caries incidence that were statistically highly significant were again observed. However, the dental caries incidences of the rats in these subgroups were significantly higher than the dental caries incidences of the rats in the earlier subgroups where higher levels of supplementation had been followed. In subgroups G and H, the rats

fed the chocolate dairy supplement had lower dental caries incidences than those fed the plain dairy supplement.

As in the filial generation of the first experiment, no developmental benefit to the offspring with respect to susceptibility to dental caries was observed as a result of the feeding of the females in the maternal generation with the plain or chocolate dairy supplements at levels to provide 45% of the Caloric requirements.

DISCUSSION

The use of mineralized milk as the sole source of nutrients for caries-susceptible cotton rats and Norway rats throughout the entire post-eruptive experimental period has been shown to result in practically the complete prevention of dental caries (Schweigert et al., '46; Shaw, '50). In the current experiments, the consumption of dairy supplements was maintained in general at lower levels and altered in relation to the stage in the life history of the experimental subjects. Both the post-eruptive and developmental influences of the consumption of dairy products were studied. The levels of consumption were chosen with the desire to approximate desirable levels of ingestion of dairy products by the human population at different ages. Hence the levels of Caloric consumption of 30% from milk, of 30% from chocolate drink or 35% from chocolate milk were chosen for the breeding females of the maternal generation in the first experiment. The choice of 45% as the level for the Caloric intake of the dairy supplements for the breeding females in the second experiment provided approximately 30% of the Calories from milk or chocolate milk, 10% from ice cream and 5% from cheese.

Among the weanlings in the filial generation, the dairy products were fed as the exclusive source of nutrients during the first 6 weeks of the post-weaning period in the first experiment and for the first three weeks in the second experiment. These periods were considered to be rough approximations of the early post-weaning period in children. After either the 6- or

three-week period with the exclusive consumption of dairy products, the amount of Calories provided for dairy products was reduced to either 33 or 25% for the duration of the experimental period.

Discussion of the results of these experiments needs to be clearly segregated into two areas:

1. Those results that occurred as a result of influences exerted during the post-eruptive period in the tooth's life history. Then the major influences in all probability make themselves manifest through the oral environment, presumably by reductions in the amount of food retained on the caries-susceptible surfaces of the teeth or by otherwise altering the oral environment to reduce its potentiality to cause the initiation and progression of carious lesions.

2. Those results that occurred as a result of influences exerted during the developmental period of the tooth's history but which are not necessarily manifest until the tooth has erupted and has been exposed to the oral environment. In this case, the influences probably operate through the development of teeth with an altered inherent susceptibility to dental caries.

Irrespective of the dairy product (milk, chocolate drink or chocolate milk or the mixtures of milk, vanilla ice cream and cheddar cheese or of chocolate milk, vanilla ice cream and cheddar cheese) used under the above circumstances in those post-eruptive trials beginning at weaning, major, highly significant reductions in dental caries incidence occurred as a result of the post-eruptive exposure of the teeth to these dietary regimens. The only occasion on which any of these supplements did not cause significant reductions in dental caries incidence on a post-eruptive basis was among the females in the maternal generation of the second experiment. In this case, supplementation was begun late in life after high dental caries incidences had already occurred when little influence could be expected to be exerted by any supplement.

There was a general trend for the dairy supplements of chocolate drink, chocolate milk and the mixture containing chocolate milk, vanilla ice cream and cheddar cheese to have been slightly less effective than the supplements of milk and of the mixture of milk, vanilla ice cream and cheddar cheese. Although no specific studies were conducted in this series to determine the reason for this difference, one would tend to suspect the reason for these differences to be the increased carbohydrate content of the preparations containing chocolate rather than chocolate itself. It should be emphasized that the difference between the various dairy products outlined above is trivial in comparison with the major, highly significant reductions resulting on a post-eruptive basis from the consumption of both plain and chocolate-flavored dairy products under the conditions in these experiments.

The early post-weaning period when the teeth have recently erupted into the oral cavity and are attaining their full maturity appears to be a particularly important time in so far as the post-eruptive feeding of dairy products is concerned. In the second experiment where the two mixtures of dairy products were compared, the exclusive feeding of these mixtures for the first three weeks of the experimental period appears to have aided post-eruptively in the establishment of a degree of caries-resistance. This influence is evidenced by the fact that the feeding of the regimen in which 33% of the Caloric requirement was supplied by the dairy product supplements and 67% by the cariogenic diet did not cause any appreciably higher caries incidence when fed for 85 days afterwards than for 64 days. To a lesser extent, this influence was observed when the regimen of 25% of Calories from the dairy product supplements and 75% from the cariogenic diet was followed for 85 days instead of for 64 days.

With respect to developmental influences upon the teeth, none of the dairy products appeared to exert any influence under the conditions prevailing in these experiments. In other words, the teeth of the rats in the filial generation did not have

any different susceptibility to dental caries when their mothers received a dairy product supplement throughout pregnancy and lactation than when their mothers received the basal rations exclusively. Since the basal rations contained all known essential nutrients, organic and inorganic, in generous amounts, the dairy products can be said to have supplied the necessary nutrients for the development of teeth at least as well as the basal rations. However, no unique characteristics were detected about the dairy products to indicate that supplements of them under the conditions of this experiment had any ability to promote the formation of more caries-resistant teeth in circumstances where all known nutrients already were supplied adequately. On the basis of current knowledge, it is impossible to state whether supplementation of partially deficient, or unbalanced diets by comparable levels of dairy products might have led to other results.

In the groups of rats which received supplements of dairy products post-eruptively, whose mothers had also received the same dairy product through pregnancy and lactation, there was no evidence that they received a greater benefit with respect to dental caries as a result of the availability of the dairy product through both periods than the rats who received the dairy supplement post-eruptively only.

The application of these studies to human populations cannot be predicted with any certainty until comparably controlled clinical studies are conducted. Longer time intervals and equally rigorous control circumstances will be needed in clinical studies because of the long period in the growth and development of the individual during which the teeth are developing and erupting into the oral cavity.

Little has been done in the field of caries research to test the influence of various human foods as supplements to a cariogenic dietary regimen either post-eruptively or developmentally. The experimental designs used in these studies, particularly the design in the second experiment, are suggested as possible ones for the extensive testing of the influ-

ence of individual foods and groups of foods during the development and post-eruptive maintenance of the teeth. Control situations are used extensively in these experimental designs in the desire to reduce the possibility that any unrecognized outside variables might influence the validity of the results. One of the chief obstacles in the design of these studies is the late stage of development of the molars of the rats at weaning. Practically the entire dietary influence during the development of the tooth has to be exerted through the mother with the concomitant barriers of the placenta during pregnancy and the mammary gland during lactation.

SUMMARY AND CONCLUSIONS

A total of 669 rats of the Harvard caries-susceptible strain was used in two experiments to test the post-eruptive and developmental influence of the supplementation of a cariogenic regimen with either milk, chocolate drink, chocolate milk, a mixture of milk, vanilla ice cream and cheddar cheese, or a mixture of chocolate milk, vanilla ice cream and cheddar cheese. The levels of supplementation varied with the age of the experimental subject and were chosen to approximate levels of human consumption.

All supplements of dairy products caused major reductions in the incidence of dental caries when fed on a post-eruptive basis only.

None of the supplements caused any detectable influence on the development of the teeth to alter their caries susceptibility.

When the supplements were provided continuously through both the developmental and post-eruptive periods, there was no demonstrable supplementary effect beyond the major influence caused by post-eruptive supplementation alone.

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EFFECT OF DIET ON THE DEVELOPMENT OF BETA-AMINOPROPIONITRILE-INDUCED VASCULAR HEMORRHAGE IN TURKEYS [1]

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Barnett, Bird, Lalich and Strong ('57) reported that the addition of 0.03 to 0.06% of beta-aminopropionitrile (BAPN), in the form of the hydrochloride, to the diet of young turkeys resulted in massive hemorrhage of the aorta or heart prior to 5 weeks of age. Waibel and Pomeroy ('58) extended this report to indicate that the feeding of lower levels of BAPN to turkeys delayed the onset of hemorrhage to 10 to 12 weeks of age, and produced aortic rupture and lesions which were grossly indistinguishable from those found under field conditions. This condition has been described by McSherry et al. ('54), Carnaghan ('55), and Gibson and de Gruchy ('55).

The cause of the occurrence of dissecting aneurysm under field conditions is unknown. A recent survey [2] conducted by the authors among Minnesota turkey growers showed that various strains of Broad-Breasted Bronze and Broad-Breasted White turkeys are susceptible to the hemorrhage, while the smaller types of turkeys are rarely affected. Further, turkeys receiving various management and feeding programs (e. g., litter vs. wire floor brooding, range vs. confinement raising, all-mash vs. concentrate and grain feeding programs,

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[2] Unpublished data.

and various medications) appeared equally susceptible to the disorder.

The object of the research reported herein was to examine the effect of diet formulation on the incidence of hemorrhage induced by feeding BAPN. Dietary BAPN was employed at a level calculated to produce death after a period of time. Thus any variations in the onset of death were attributed to diet modifications.

EXPERIMENTAL

Broad-Breasted Bronze or White turkey poults were used in all experiments. They were housed during the first three weeks in electrically heated batteries with raised wire-mesh floors. After this time they were moved to conventional growing batteries containing raised wire-mesh floors. Details showing breed, number of birds per group, and experimental duration are given in the tables.

Variables of ration formulation are shown in the tables along with calculated protein and energy values. The ratio of productive energy calories (Fraps, '46) to protein was held constant, except when under study. Feeding ingredients common to all experimental groups within each experiment are shown in table 1. Beta-aminopropionitrile [3] was obtained as the fumarate.

All fish meal samples were obtained on the open market. Fish meal no. 1 was specified Menhaden and analyzed 59.2% protein, 5.7% fat, 1.8% fiber, 23.4% ash, 7.4% moisture, 7.4% calcium and 4.0% phosphorus. Fish meal no. 2 was unspecified as to source and analyzed 52.9% protein, 18.1% fat, 8.2% calcium and 4.4% phosphorus. Fish meals 3, 4, and 5 were unspecified as to source and were guaranteed to contain a minimum of 60% protein. Fish meal no. 6 was specified Herring and guaranteed to contain a minimum of 70% protein.

Turkeys succumbing during the course of the experiment were examined for cause of death. In experiment 1 (table 2)

[3] The authors are grateful to Dr. D. V. Frost, Abbott Laboratories, North Chicago, Ill., for supplying the beta-aminopropionitrile fumarate.

TABLE 1

Constant dietary components within each experiment

INGREDIENT	EXPERIMENT		
	1	2	3
	%	%	%
Fish solubles [1]	3	—	—
Dried whole whey	3	3	—
Alfalfa meal	3	3	—
DL-Methionine	—	—	0.1
Salt, iodized	0.3	0.3	0.5
Manganese sulfate, feed grade	0.025	0.025	—
Trace mineral mixture [2]	—	—	0.15
Vitamin A (10,000 I.U./gm)	0.05	0.05	0.1
Vitamin D (3000 I.C.U./gm)	0.05	0.05	0.05
Vitamin E (20,000 I.U./lb.) [3]	0.025	0.025	0.05
Menadione sodium bisulfite, 63% (4 gm/lb.) [4]	0.025	0.025	—
Vitamin mixture 93 [5]	0.1	0.1	—
Vitamin mixture C-5 [6]	—	—	0.5
Choline chloride (25%)	0.1	0.1	0.15
Vitamin B_{12} (10 mg/lb.)	0.1	0.1	—
Procaine penicillin (4 gm/lb.)	0.1	0.1	0.1

[1] Fish solubles supplied as Dynasol 100S. One hundred per cent equivalent of fish solubles dried on soybean oil meal. Vylactose Laboratories, Des Moines, Iowa.

[2] Trace minerals supplied as Delamix. Trace mineral premix containing 6% manganese, 0.12% iodine, 2% iron, 0.2% copper, 0.006% zinc, 0.02% boron, and 27% calcium. Limestone Products Corp., Newton, N. J.

[3] Vitamin E supplied as *d*-alpha tocopheryl acetate in Myvamix. Distillation Products Industries, Rochester, N. Y.

[4] Menadione sodium bisulfite, 63% supplied as Klotogen F Premix (4 gm/lb.). Abbott Laboratories, North Chicago, Illinois.

[5] Vitamin mixture 93 contains 4 gm riboflavin, 4 gm calcium pantothenate, 24 gm niacin and 104 gm choline chloride per pound. Merck and Co., Rahway, N. J.

[6] Vitamin mixture C-5 supplies the following per 5 grams (corn meal diluent): 0.2 mg biotin, 2 mg menadione sodium bisulfite-63%, 2 mg pyridoxine·HCl, 2 mg folic acid, 2 mg thiamine·HCl, 5 mg riboflavin, 10 mg calcium pantothenate, 40 mg niacin, 30 μg vitamin B_{12} (as 0.1% trituration of cyanocobalamin with mannitol), and 200 mg Cerelose.

all surviving birds were subjected to necropsy and condition of the posterior aorta at the usual point of rupture near the external iliac artery was recorded. If no gross lesions were noted, the aorta was considered normal. Dilated and thickened aortas and the presence of yellowish plaques on the intima of the aorta were also noted.

Hemorrhage severity index. The rapidity with which death due to hemorrhage occurred was considered the major response to diet variations. The following formula was used to calculate the severity index:

$$\text{Index} = \frac{(\text{DDH in A} \times 1) + (\text{DDH in B} \times 2) + (\text{DDH in C} \times 3) \text{ etc.}}{(\text{birds started} - \text{non-hemorrhaging mortality})/\text{birds started}}$$

Where:

DDH = Death due to hemorrhage
A = Last period of experiment (exp. 1, 56 to 69 days of age; exps. 2 & 3, 23 to 28 days).
B = Period preceding A (exp. 1, 43 to 55 days; exp. 2 & 3, 17 to 22 days).
C = Period preceding B (exp. 1, 28 to 42 days; exps. 2 & 3, 11 to 16 days).
D = Period preceding C (exp. 1, 14 to 27 days; exp. 2 & 3, zero to 10 days).
E = Period preceding D (exp. 1, 4 to 13 days of age).

This index gives necessary importance to time of hemorrhage. It assumes that birds which died of "other" causes were not a part of birds eligible for hemorrhage expression. This is largely true since most of these losses occurred prior to 10 days of age.

Leg weakness index. To record leg weakness symptoms caused by BAPN, each bird was observed and recorded for locomotion agility at two weeks of age. Birds walking normally were scored zero, birds showing shaky or slow locomotion were scored one, and birds which staggered or were down on their hocks scored two. These scores were averaged and are shown in the tables.

RESULTS

All groups in experiment 1 were fed 3% of fish solubles, 3% of dried whole whey and 3% of alfalfa meal in addition to necessary supplements of vitamins and minerals (table 1). Other diet variables are shown with the experimental data in table 2. All 10 control birds (group 1) survived with normal appearing aortas at 69 days of age. The hemorrhage severity index indicates a wide range of mortality expression in birds receiving 0.04% of BAPN. Very little mortality occurred in groups 2, 5, and 9 (BAPN control, high protein with corn gluten meal, and 4-nitrophenylarsonic acid, respectively). However, with the use of 15% of fish meal (group 3), all birds

TABLE 2

Effect of dietary variables on growth and incidence of beta-aminopropionitrile (BAPN)-induced hemorrhage in turkeys (experiment 1)[1]

	GROUP NUMBER									
	1	2	3	4	5	6	7	8	9	10
Variable formulation (% in diet):										
Ground yellow corn	41.8	41.8	43.8	43.5	26.8	12.2	12.2	12.2	41.8	41.8
Soybean oil meal, dehulled	43	43	28.5	31	43	57.5	57.5	57.5	43	43
Dicalcium phosphate	3	3	0.75	—	3	3	3	3	3	3
Calcium carbonate	2.25	2.25	2	0.50	2.25	2.25	2.25	2.25	2.25	2.25
DL-Methionine	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1
Fish meal (Menhaden) No. 1 [1]	—	—	15	—	—	—	—	—	—	—
Meat and bone meal (50% protein)	—	—	—	15	—	—	—	—	—	—
Corn gluten meal	—	—	—	—	15	—	—	—	—	—
Tallow, bleachable fancy	—	—	—	—	—	15	—	—	—	—
Corn oil, refined	—	—	—	—	—	—	15	—	—	—
Soybean oil, crude	—	—	—	—	—	—	—	15	—	—
4-Nitrophenylarsonic acid [2]	—	—	—	—	—	—	—	—	0.025	—
Ascorbic acid	—	—	—	—	—	—	—	—	—	0.20
beta-Aminopropionitrile fumarate	—	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Calculated analysis:										
Protein, %	28.1	28.1	30.2	29.7	33.3	33.1	33.1	33.1	28.1	28.1
Productive energy (Fraps) Cal./lb.	797	797	864	850	749	964	964	964	797	797
Performance:										
Weight, 27 days, gm	460	448	433	497	517	512	541	547	497	570
Weight, 69 days, gm	2438	2222	—	1852	2172	2587	2544	2456	2054	2362
Hemorrhage index (See experimental)	0	1	31	11	3	7	11	6	0	10
Survivors, 69 days	10	8	0	3	8	6	5	6	7	4
Autopsy — no lesions in aorta	10	2	—	0	4	1	1	2	5	2
Autopsy — dilated and thickened aorta	0	6	—	3	4	4	4	4	2	2
Autopsy — yellow plaque on intima	0	5	—	3	1	3	3	1	2	2

[1] Broad-Breasted White turkeys, 10 males per group. Newly hatched poults were placed on diet 1 for 5 days prior to equalized segregation into experimental lots. Experiment terminated at 69 days of age.

[2] 4-Nitro phenylarsonic acid supplied in Histostat, Dr. Salsbury's Laboratories, Charles City, Iowa.

had succumbed by 35 days of age. Groups 4, 6, 7, 8, 10 (meat and bone meal, animal fat, corn oil, soybean oil, and ascorbic acid, respectively) showed greater mortality than did the BAPN controls (group 2), but were much less affected than the birds receiving fish meal.

A 0.08% level of BAPN was utilized in all treated groups in experiment 2 (table 3) thus the onset of hemorrhaging was hastened. All groups in this experiment were fed 3% of dried whole whey and 3% of alfalfa meal as constant dietary components (table 1). Each group receiving BAPN had a control without BAPN. The control birds did not hemorrhage or develop leg symptoms. Their weights and efficiencies are given. The hemorrhage severity index shows that the substitution of 3% of fish meal for 3% of fish solubles (groups 1–2) was sufficient to hasten the hemorrhage expression. Triiodo-L-thyronine (group 3) did not retard hemorrhage. With group 2 serving as 3% fish meal control, it is apparent that 15% each of beef tallow, hog grease, soybean oil or safflower oil (groups 4 to 7) did not hasten hemorrhage. Rather, the animal fats may have alleviated it somewhat. In treated groups 8, 9, and 10, the use of 15% of two samples of fish meal and meat and bone meal, respectively, produced intermediate hemorrhage indexes. In this experiment, 15% of fish meal no. 1 (group 8) produced a high degree of mortality during the second and third weeks, but on necropsy massive internal hemorrhage could not be established as the cause of death. This same fish meal sample has been used in 4 other experiments with a high yield of definite aortic or heart ruptures.

The results obtained in experiment 3 are reported in table 4. Only one non-treated control group was maintained together with 19 BAPN groups, to allow greater exploration of the effects of dietary variables. The group without BAPN received 15% of fish meal no. 1 and performed quite normally, indicating that in these studies fish meal had no hemorrhage-producing effect in the absence of BAPN.

TABLE 3

Effect of protein supplements and fats on beta-aminopropionitrile (BAPN)-induced hemorrhage in turkey poults. (experiment 2)[1]

	GROUP NUMBER									
	1	2	3	4	5	6	7	8	9	10
Variable formulation (% in diet):										
Ground yellow corn	41.2	41.8	41.8	12.2	12.2	12.2	12.2	43.2	42.5	43
Soybean oil meal, dehulled	43	43	43	57.5	57.5	57.5	57.5	28.5	31	31
Dicalcium phosphate	3	2.5	2.5	3	3	3	3	0.75	—	—
Calcium carbonate	2.5	2.75	2.75	2.25	2.25	2.25	2.25	2.5	1.5	1
DL-Methionine	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.1	0.1	0.1
Fish solubles (Dynasol 100S)	3	—	—	—	—	—	—	3	3	3
Fish meal (Menhaden) No. 1	—	3	3	3	3	3	3	15	—	—
Fish meal (unspecified) No. 2	—	—	—	—	—	—	—	—	15	—
Meat and bone meal (50% protein)	—	—	—	—	—	—	—	—	—	15
All beef tallow	—	—	—	15	—	—	—	—	—	—
All hog grease	—	—	—	—	15	—	—	—	—	—
Soybean oil, refined	—	—	—	—	—	15	—	—	—	—
Safflower oil, refined	—	—	—	—	—	—	15	—	—	—
L-Triiodothyronine [2]	—	—	+	—	—	—	—	—	—	—
Calculated analysis:										
Protein, %	28.0	28.4	28.4	33.6	33.6	33.6	33.6	30.2	29.6	27.8
Productive energy (Fraps) Cal./lb.	785	802	802	968	968	968	968	852	861	821
Performance — controls:										
Weight 3 weeks, gm	374	359	327	409	472	506	408	416	423	392
Gain/feed	1.71	1.67	2.36	1.50	1.33	1.28	1.40	1.52	1.54	1.74
Performance — 0.08% BAPN:										
Weight 3 weeks, gm	340	308	255	340	353	401	339	263	325	296
Hemorrhage index (See experimental)	6	21	21	7	10	19	16	15	14	12
Survivors at 28 days	7	2	2	6	7	0	3	2	4	5
Leg weakness index (See experimental)	1.33	0.91	0.86	0.45	1.0	1.0	0.82	1.50	1.18	1.36

[1] Broad-Breasted Bronze turkeys, 6 males and 6 females per group. Experimental period: controls zero to 21 and BAPN-treated zero to 28 days of age.

[2] Triiodo-L-thyronine manufactured by Smith, Kline, and French Laboratories, Philadelphia, Pa., under trade name "Cytomel." Level employed 2.5 mg L-triiodothyronine per kilogram diet.

In groups 2 to 8, varying fish meal sources and levels were employed and the hemorrhage severity index ranged from 13 to 22. In groups 11, 13, and 16 to 20 no fish meal was present and the variables included corn-soybean meal types of rations with and without such feeding ingredients as wheat bran, wheat standard middlings, ground oats, alfalfa meal, and dried whey. In these groups the hemorrhage index ranged from zero to 6. Inspection of the time of mortality due to hemorrhage in these various groups indicates clearly that the fish meal samples hastened the onset of hemorrhage compared to the groups not receiving fish meal.

In groups 9 and 10 a defatted fish meal and cod liver oil, respectively, were fed in order to obtain possible information as to the constituent of fish meal responsible for the onset of hemorrhaging. The results in this and in a further unreported experiment suggested that hemorrhage appeared earlier in the group receiving defatted fish meal. The use of 5% of cod liver oil with BAPN produced a low hemorrhage index, but mortality due to other causes was higher.

In group 12 (low protein) mortality from hemorrhaging seemed to be speeded up somewhat. No attempt has been made to repeat this observation. Due to a popular feeling that commercial birds suffering from hemorrhage recovered when fed increased levels of dicalcium phosphate, a group was included in this experiment wherein calcium and phosphorus deficiency was instituted by using levels of 1.27% calcium and 0.70% phosphorus in contrast to normal 2.0% and 1.0% levels, respectively, fed in turkey starting rations. The onset of hemorrhage did not appear to be hastened. However, three birds in the group suffered from a condition known as spraddles, wherein both legs extend from the body and the bird cannot stand. This was thought to be due primarily to a deficiency of calcium or phosphorus.

In experiments 2 and 3, record was made of the degree of leg weakness observed in poults receiving BAPN. In experiment 2, no great trends were observed between treatment and

TABLE 4

Effect of various feedstuffs on beta-aminopropionitrile (BAPN)-induced hemorrhage in turkey poults (experiment 3)[1]

DIET VARIABLES (%) AND RESULTS	GROUP NUMBER									
	1	2	3	4	5	6	7	8	9	10
Ground yellow corn	47.6	46	47.6	50.3	47.6	47.6	47.6	50	52.3	35.3
Soybean oil meal, dehulled	32.5	42	32.5	23	32.5	32.5	32.5	30	27.5	52
Dicalcium phosphate	0.75	2.25	0.75	—	0.75	0.75	0.75	0.75	1	3
Calcium carbonate	2.5	3	2.5	—	2.5	2.5	2.5	2.5	2.5	3
Fish meal (see experimental)	15(#1)	5(#1)	15(#1)	25(#1)	15(#3)	15(#4)	15(#5)	15(#6)	—	—
Defatted fish meal [2]	—	—	—	—	—	—	—	—	15	—
Cod liver oil	—	—	—	—	—	—	—	—	—	5
beta-Aminopropionitrile fumarate	—	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Calculated analysis:										
Protein, %	30.1	29.6	30.1	31.6	30.1	30.1	30.1	30.2	31.0	29.7
Productive energy (Fraps) Cal./lb.	893	848	893	942	893	893	893	903	907	878
Performance:										
Weight 2 weeks, gm	242	193	201	175	224	175	200	191	235	157
Hemorrhage index (see experimental)	0	13	20	21	13	22	17	18	11	3
Survivors at 4 weeks	12	3	3	2	4	2	0	1	5	6
Leg weakness index (see experimental)	0	0.27	1.55	1.84	0.58	1.25	0.42	1.17	0.44	0.0

DIET VARIABLES (%) AND RESULTS	GROUP NUMBER									
	11	12	13	14	15	16	17	18	19	20
Ground yellow corn	45.3	54.7	31.3	47.8	47	34.3	34.3	28.3	41.6	38.6
Soybean oil meal, dehulled	47	37.6	61	47	35	38	38	44	41	44
Dicalcium phosphate	3	3	3	1.5	—	2.5	2.75	3	3	2.75
Calcium carbonate	3	3	3	2	1.25	3.5	3.25	3	2.75	3
Meat and bone meal (50% protein)	—	—	—	—	15	—	—	—	—	—
Wheat bran	—	—	—	—	—	20	—	—	—	—
Wheat standard middlings	—	—	—	—	—	—	20	—	—	—
Ground oats	—	—	—	—	—	—	—	20	—	—
Alfalfa meal	—	—	—	—	—	—	—	—	10	—
Dried whey	—	—	—	—	—	—	—	—	—	10
beta-Aminopropionitrile fumarate	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Calculated analysis:										
Protein, %	28.0	23.9	33.9	28.2	29.6	25.7	26.0	27.4	26.4	27.1
Productive energy (Fraps) Cal./lb.	823	864	742	851	879	736	742	772	765	772
Performance:										
Weight 2 weeks, gm	187	204	184	189	199	178	185	207	190	174
Hemorrhage index (see experimental)	4	12	5	4	16	4	1	6	2	0
Survivors at 4 weeks	7	3	8	7	3	7	11	8	9	11
Leg weakness index (see experimental)	0	0.17	0.30	0.82	0.08	0.25	0.08	0.08	0.27	0

[1] Broad-Breasted Bronze turkeys, 12 males per group. Experimental period: zero to 28 days of age.
[2] Viobin Corporation, Monticello, Illinois. Analysis: 82.7% protein, 14.3% ash, 2.8% moisture, and 0.06% fat.

index, particularly where 3% of fish meal was used. In experiment 3 the leg weakness index generally showed greater severity in the presence of 15 or 25% of fish meal (groups 3 to 9), in contrast to the lower level of fish meal (group 2). The other groups, except low calcium and phosphorus (no. 14), showed considerably less leg weakness.

DISCUSSION

It cannot be stated that any of the ingredients employed have a protective effect against the hemorrhaging. Promising results cannot be considered real on the basis of these experiments because retesting was not done. Much of these data were included in this paper to utilize a collective comparison against the effect of nocuous ingredients.

This study was designed to examine whether dietary components may influence hemorrhage incidence caused by BAPN under laboratory conditions. There is no justification for translating such findings to commercial practice. The results of the Minnesota survey (see introduction) indicate that other non-dietary factors must be involved in the field cases of aortal rupture. If diet were solely responsible for the problem, feed manufacturers would be expected to have the problem in many areas at the same time. This has not proven to be the case; that is, certain turkey growers have more difficulty with the problem than others. It is quite possible that in the presence of certain stresses, dietary factors may become important. It is planned to conduct field trials wherein certain dietary modifications are measured. Fish meal has been a very valuable ingredient in turkey diets. It would be indeed unfortunate to implicate this valuable product with any practical problem in the absence of direct evidence.

In experiment 2, a control group was present for each BAPN-treated group. In the other experiments only one non-BAPN control group was maintained and either 9 or 19 treated groups were utilized. Obviously, any important effects found in the absence of a complete control design must be repeated.

The advantage of using only one non-BAPN control is that many additional comparisons may be utilized in the same facilities.

It has been observed in these and other studies (Waibel and Pomeroy, '58) that the faster growing birds *within a group* succumb to hemorrhage at an earlier age than the slower growing members. Thus, effects of dietary changes affecting growth primarily should not be interpreted as hastening hemorrhage. In table 2, birds succumbing at an early age due to the presence of fish meal were not heavier at two or 4 weeks of age than were those in other groups in the experiment. Inspection of the various growth and mortality data does not indicate an interaction which might alter the major results.

The production of leg weakness in the studies with BAPN is of interest because of the problem turkey growers occasionally have with leg weakness in their flocks. When low dietary levels of BAPN are employed the leg weakness problem tends to remain minor enough so that growth is not greatly affected. It appeared in these experiments that higher levels of fish meal with BAPN also aggravated the leg weakness problem. It was observed in experiment 1 that at two weeks of age the group which later succumbed to fish meal showed considerably more leg weakness and enlarged hock difficulty than did the other groups.

Various compounds containing free amino groups e.g. beta-aminopropionitrile, aminoacetonitrile, beta-mercaptoethylamine, and semicarbazide have lathyrogenic activity for the rat (Dasler, '58). Certain protein sources and amino acids have been shown effective in minimizing growth depression and skeletal change induced by feeding lathyrogenic diets to rats (Lee et al., '56; Dasler, '54 and '56). Bruemmer et al. ('57) suggest that BAPN effects amino acid incorporation into collagen or ground substance rather than the cellular portion of connective tissue. Thus the evidence suggests an important relationship between amino acid metabolism and the development of lathyrism. The present results suggest

that increasing the protein level with corn gluten meal or soybean oil meal (experiments 2 and 3) does not aggravate the onset of hemorrhage. It is tempting to speculate on the presence of amino compounds in certain fish meal and meat meal samples, but this will be omitted in deference to experiments which provide an answer.

The well known influence of dietary fats on blood cholesterol levels and the occurrence of yellowish plaques in the intimal layer of the aortas of turkeys treated with BAPN suggested that the nature of dietary fat might have some importance in the production of dissecting aneurysms. Thus, in tables 2 and 3 are indicated various groups receiving 15% of various sources of animal and vegetable fats. The nature of dietary fat had no marked influence upon the development of hemorrhage. This supports the observation of Hukill ('57) that essentially all cases of dissecting aneurysm occurring in the aortas of humans are accompanied by cystic medial necrosis and that dissection bears no relation to intimal disease and arteriosclerosis. Although conditions are not comparable, our findings showing no effect of added fat on BAPN-induced hemorrhage are at variance with those of Pritchard et al. ('57), where turkeys receiving animal fat and no BAPN appeared to suffer more hemorrhage due to dissecting aneurysms of large vessels.

The use of fat in these experiments raises the very important question of providing a uniform dosage of BAPN. An approximate 20% increase in dietary energy was obtained by the addition of 15% of fat (using a value of 2760 Cal. of productive energy for the various fat sources). Obviously, the addition of energy to a balanced diet decreases feed and therefore BAPN consumption. The present results have been studied to detect whether such energy changes alter the meaning of the experimental findings. It is not believed that major effects of this kind are operative.

Since low doses of triiodo-L-thyronine suppressed mild forms of aminoacetonitrile poisoning in rats (Ponseti, '57), it was tested as a possible antidote to BAPN toxicity in

turkeys. The single level employed (2.5 mg/kg of diet) was used successfully by Ponseti. There did not appear to be any antidotal action on the part of this compound. Further studies with turkeys would be in order, however, to establish proper dosage levels.

An intriguing possibility is that animal products may exert an effect through hormonal action. It has been demonstrated by Selye and Bois ('57) that somatotropic hormones greatly aggravate skeletal manifestations of experimental lathyrism produced with aminoacetonitrile. These workers cite also that desoxycorticosterone and cortisol in combined treatment can completely reverse the normal morphological syndrome of experimental lathyrism in that this corticoid combination causes aminoacetonitrile to produce aortal aneurysm in the virtual absence of bone lesions in rats. The finding under field conditions that aortic rupture in turkeys occurs primarily in males may be more profund than merely the effect of faster rate of growth. Alteration of the relatively high blood pressure of male turkeys (Weiss and Sheahan, '58) may also be an important means of hastening or retarding aortic hemorrhage.

SUMMARY

Levels of 0.04 to 0.08% of beta-aminopropionitrile fumarate (BAPN) were administered to turkeys in a diet composed mainly of ground corn and soybean oil meal in order to produce eventual death due to aortal and heart hemorrhage.

Under these conditions, certain formula modifications were tested for their capacity to alter hemorrhage incidence. Six fish meal samples at a 15% dietary level were found to hasten the appearance of BAPN-induced mortality. In the absence of BAPN, fish meal produced excellent growth.

Feeding ingredients which did not appear to hasten the onset of hemorrhage due to BAPN included: all-beef tallow, all-hog grease, soybean oil, corn oil, or safflower oil, each at the 15% dietary level, wheat standard middlings, wheat bran, or ground oats at the 20% level, or alfalfa meal or dried whole whey at the 10% level.

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DIET AND CHOLESTEREMIA

I. DEVELOPMENT OF A DIET FOR THE STUDY OF NUTRITIONAL FACTORS AFFECTING CHOLESTEREMIA IN THE RAT [1]

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The causes of atherosclerosis are not known. Its development is thought to be associated with defects in lipid metabolism and with hypercholesteremia of dietary or metabolic origin. Current interest in this problem has been focussed on nutritional factors which affect the level of cholesterol in blood. Among the factors that have been studied, either in man or in experimental animals, are the kind and level of dietary fat and protein, the amount of cholesterol and plant sterols in the diet, the intake of pyridoxine, choline, nicotinic acid, sulfur-containing amino acids and magnesium. The exact roles of these factors have not been established and reports about their effects are not always in agreement. The present investigation was, therefore, undertaken in an attempt to develop a diet that would make it possible to study, under controlled conditions, the effects of various nutritional factors on serum cholesterol concentration in the rat.

The rat is quite resistant to the development of atherosclerosis. However, Fillios et al. ('56) were able to produce gross atherosclerotic lesions in the rat and while this study was in progress, Hegsted et al., ('57) reported that vascular

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sudanophilia was proportional to serum cholesterol level with a variety of dietary fats. If sudanophilia be taken as a measure of early "atherogenic activity," the cholesterol level of the serum should reflect this activity; thus the rat may be as useful as other species for the study of dietary factors affecting the development of atherosclerosis.

Observations on the effects of (a) hydrogenated coconut oil, with and without the addition of corn oil, (b) pyridoxine deficiency and (c) dietary additions of cholesterol, cholic acid, and thiouracil on serum cholesterol concentration in the rat are reported below. In addition, the effects of these factors on growth, essential fatty acid deficiency, level of serum lipid phosphorus and total liver lipid, cholesterol and lipid phosphorus concentrations are included.

EXPERIMENTAL

Material and methods. Male weanling rats of either the Sprague-Dawley or the Holtzman strain weighing 40 to 55 gm were used in these experiments. They were housed in individual cages with screen bottoms, and were fed *ad libitum*. The rats were observed for skin irregularities and weighed weekly. Scaliness of the skin was graded on a numerical basis (Schreiber et al., '52) with normal rats being rated zero and those with very severe dermatosis, 4. Blood was drawn from individual rats under ether anesthesia by cardiac puncture after a 15 to 20-hour fasting period, except that in experiment 1 the rats were not fasted. Cholesterol and lipid phosphorus determinations were done on pooled serum samples.

At the end of the investigation blood samples were taken, then the rats were killed and the livers, hearts and aortas were removed. Livers were frozen at —18° while hearts and aortas were preserved in 10% formalin for histological examination. Livers were later analysed for total lipid, total cholesterol and lipid phosphorus. Equal weights of each liver from the rats in a single group were pooled for analysis.

Analytical procedures. Serum cholesterol was determined by the method of Sperry and Webb ('50). Serum lipid phosphorus was determined by the method of Youngburg (Hawk et al., '54). Livers were analysed for total lipids, total cholesterol and lipid phosphorus by the method used by Fillios and Mann ('54).

Diet. The basal diet contained vitamin test casein supplemented with 2% of DL-methionine, 20%; Salts IV (Hegsted et al., '41) 4%; sucrose and hydrogenated coconut oil (HCO), with or without corn oil as indicated in the tables of results. The levels of other ingredients used in the experiments such as cholesterol, cholic acid, and thiouracil, are given with the description of each experiment. All changes in the diet were compensated for by adjusting the level of sucrose. Water-soluble vitamins were fed at the following levels (milligrams per kilogram) in the diet: thiamine·HCl, 8.0; riboflavin, 6.0; pyridoxine, 4.0; calcium pantothenate, 40.0; niacin, 50.0; inositol, 200.0; folic acid, 4.0; vitamin B_{12}, 0.04; biotin, 0.20 and choline chloride 2000. β-Carotene, 400 mg; calciferol, 3 mg; menadione, 400 mg; α-tocopherol acetate, 3.0 gm were dissolved in 25 ml of absolute alcohol and three drops of the solution were administered orally per rat per week.

RESULTS

Experiment 1

The effects of different levels of hydrogenated coconut oil, with or without 1% of corn oil and cholesterol, on serum cholesterol and lipid phosphorus concentrations were studied in this experiment. Diets containing 1, 10 and 25% of HCO were used. There were 10 rats in each group and the experiment was continued for a period of 18 weeks. A few animals died as a result of heart punctures and some developed lung infections during the latter part of the experiment. Therefore, weight gains are given for only 5 and 10 weeks. Four animals from each group were killed after 7 weeks. Blood was analysed after the 4th week, then at weekly intervals for three weeks,

and later after longer periods to give a total of 6 analyses in all. The results are presented in table 1. Average values are given for serum cholesterol and lipid phosphorus.

Growth and dermatosis. When the HCO level in the diet was raised from 1 to 10%, an increase in weight gain was observed at 5 and at 10 weeks. A further increase in the HCO level to 25%, however, depressed growth. The dermatosis score decreased with each increase in the HCO level from 1 to 25%. The addition of 1% of cholesterol to the diet containing 10% of HCO depressed growth slightly and caused a slight increase in the dermatosis score after 5 weeks. Although these effects of cholesterol were not prominent they became more evident in the next two experiments.

The substitution of 1% of corn oil for 1% of the HCO in the diet prevented scaliness of the skin and increased the growth rate. There were no significant differences among the weight gains of the groups receiving 1% of corn oil. The addition of cholesterol in the presence of 1% of corn oil did not affect either the growth rate or the scaliness of the skin.

Serum constituents. Increasing the level of HCO in the diet from 1 to 25%, with or without 1% of corn oil did not affect serum cholesterol concentration (table 1). The average values with no corn oil in the diet were from 81 to 89 mg % and with 1% of corn oil were 92 to 97 mg %. The addition of 1% of corn oil caused a little elevation of the serum cholesterol concentration with each dietary level of fat. There was, however, a significant fall in serum cholesterol concentration, when 1% of corn oil was included in the diet with 1% of cholesterol; the mean value without corn oil in the diet was 336 mg % and with 1% of corn oil was 209 mg%. Serum lipid phosphorus concentration was not affected by the dietary level of fat, or by the addition of cholesterol to the diet, but tended to rise when 1% of corn oil was added to the diet.

Liver constituents. Total lipid, total cholesterol and lipid phosphorus concentrations in the livers of rats killed at the end of the experiment are given in table 1. Changing the fat

TABLE 1

Effect of dietary level of fat and of additions of corn oil and cholesterol on serum cholesterol concentration

GROUP NO.	DIET			WEIGHT GAIN		DERMATOSIS SCORE		SERUM		LIVER		
	HCO [1]	CO [1]	Cholesterol	5 wk.	10 wk.	5 wk.	10 wk.	Total cholesterol	Lipid P	Total lipids	Total cholesterol	Lipid P
	%	%	%	*gm*	*gm*			*mg %*	*mg %*	%	*mg %*	*mg %*
101	1	–	–	161 ± 4 [2]	228 ± 7 [2]	1.6	2.6	89 ± 4 [2]	4.1 ± 0.1 [2]	6.3	119	41
102	–	1	–	198 ± 2	297 ± 9	0	0	97 ± 3	4.7 ± 0.2	4.1	59	36
103	10	–	–	176 ± 2	244 ± 3	1.4	2.7	81 ± 6	4.5 ± 0.2	3.7	59	42
104	10	–	1	169 ± 3	238 ± 7	1.8	2.4	336 ± 45 [3]	4.9 ± 0.2	18.4	5140	60
105	9	1	–	197 ± 7	297 ± 10	0	0	98 ± 5	5.0 ± 0.2	5.3	126	55
106	9	1	1	197 ± 4	311 ± 6	0	0	209 ± 30 [3]	5.6 ± 0.2	6.2	3070	–
107	25	–	–	151 ± 2	213 ± 3	1.1	2.0	86 ± 5	4.7 ± 0.2	4.8	81	53
108	24	1	–	192 ± 2	289 ± 6	0	0	92 ± 6	5.2 ± 0.2	3.2	64	47

[1] HCO = hydrogenated coconut oil; CO = corn oil.

[2] Standard error of the mean (SEM).

[3] Large SEM in these groups was due to one very high value in each group.

level in the diet from 1 to 25%, with or without 1% of corn oil, did not alter the values for any of these constituents. Most of the values for total liver lipids were within the normal range except that the value for the 1% HCO group was slightly high.

The addition of 1% of cholesterol to the diet containing 10% of HCO caused the total lipids to increase to 18.4% and total cholesterol to more than 5%. The increase in liver lipids was, in part, a result of the greater cholesterol deposition. Liver lipid phosphorus was not influenced by cholesterol feeding. The addition of 1% of corn oil to the diet along with 1% of cholesterol caused a reduction in both total lipid and total cholesterol concentration. Nevertheless, the amount of cholesterol deposited in the liver was still very high compared to the values for groups not ingesting cholesterol.

Experiment 2

The effect of excluding pyridoxine from the diet was studied in this experiment. The diets contained 25% of fat and 1% of cholesterol. There were 8 animals in each group. The experiment was continued for 15 weeks. At the end of 4 weeks blood was drawn for analysis from all rats receiving sufficient pyridoxine, but from only half of those fed the pyridoxine-deficient diet. The latter were killed, then pyridoxine was added to the diet of the remaining 4 animals. The results are given in table 2.

Values for serum constituents at 4 weeks and 10 weeks are given. The values after 15 weeks were similar to those at 10 weeks. Liver analyses are for rats killed at the end of 15 weeks.

Growth and dermatosis score. Comparing group 201 with 202, and 205 with 206, it is evident that the addition of 1% of cholesterol to the diet containing 25% of HCO increased the dermatosis score and depressed growth, as was noted earlier with rats fed diets containing 10% of HCO. The addition of 1% of corn oil increased the growth rate of rats on pyridox-

TABLE 2

Effect of pyridoxine deficiency on serum cholesterol concentration

GROUP NO.	DIET				WEIGHT GAIN		DERMATOSIS SCORE		SERUM				LIVER		
	HCO [1]	CO [1]	Cholesterol	Vitamin B_6	4 wk.	10 wk.	4 wk.	10 wk.	Total cholesterol		Lipid P		Total lipids	Total cholesterol	Lipid P
									4 wk.	10 wk.	4 wk.	10 wk.			
	%	%	%		*gm*	*gm*			*mg %*	*mg %*	*mg %*	*mg %*	%	*mg %*	*mg %*
201	25	–	–	+	139 ± 4 [2]	245 ± 13 [2]	0.8	2.3	62	74	4.0	4.3	5.1	97	66
202	25	–	1	+	141 ± 2	226 ± 5	1.2	2.9	168	412	4.1	6.4	15.2	4140	64
203	24	1	–	+	165 ± 3	307 ± 9	0	0	67	100	4.4	5.1	4.7	93	59
204	24	1	1	+	172 ± 4	306 ± 16	0	0	118	145	5.2	5.2	16.6	2220	57
205 [3]	25	–	–	—	35 ± 3	158 ± 10	3.0	2.4	86	72	6.7	5.2	4.7	109	51
206 [3]	25	–	1	—	26 ± 2	124 ± 2	3.5	3.2	194	336	5.4	5.2	14.2	4840	45
207 [3]	24	1	–	—	33 ± 2	201 ± 7	2.0	0	107	86	5.4	5.0	4.9	92	62
208 [3]	24	1	1	—	37 ± 5	192 ± 8	2.5	0	141	141	4.6	5.3	13.1	1540	82

[1] HCO = hydrogenated coconut oil; CO = corn oil.

[2] Standard error of the mean.

[3] After 4 weeks, half the animals in these groups were killed for the collection of blood samples. Pyridoxine was then added to the diets of the remainder. Liver analyses for the latter half are given in the table.

ine-sufficient diets, but was without effect on the growth of those fed the deficient diets. The dermatosis score for vitamin B_6-deficient rats was high compared to that for vitamin B_6-sufficient rats. The addition of 1% of corn oil completely prevented the dermatosis in vitamin B_6-sufficient but not in vitamin B_6-deficient animals; however, the score for the latter was lower than that for rats fed the diet lacking both corn oil and pyridoxine. Scaliness completely disappeared in 4 weeks after the addition of pyridoxine to the diet of rats receiving 1% of corn oil and 24% of HCO. Scaliness of the animals in the 25% HCO group also improved on the addition of pyridoxine to the diet. The rate of improvement of scaliness in the group fed the diet containing 25% of HCO and 1% of cholesterol (group 206) was slower and the dermatosis score was higher than that of group 205.

Serum constituents. The 4-week values for serum cholesterol for the vitamin B_6-deficient groups tend to be higher than those for the corresponding vitamin B_6-sufficient groups. There was a similar trend in serum lipid phosphorus but it was not so consistent. The addition of 1% of corn oil without exogenous cholesterol tended to increase the serum cholesterol level, but in the presence of 1% of exogenous cholesterol it caused a marked lowering of the serum cholesterol value, a finding noted in the earlier experiment.

Liver constituents. The effects on liver constituents of additions of cholesterol or corn oil to the diet were similar to those observed in the first experiment.

Experiment 3

In the two previous experiments the addition of 1% of cholesterol to the diet increased the serum cholesterol level. Therefore, this experiment was planned to determine whether a still greater elevation of serum cholesterol could be obtained in a shorter time by adding cholic acid alone or with thiouracil to the diet containing 1% of cholesterol. There were 8 groups of 8 animals each and half the animals in each group were

TABLE 3

Effect of cholesterol, cholic acid and thiouracil in the diet on serum cholesterol concentration

GROUP NO.	DIET					WEIGHT GAIN		DERMATOSIS SCORE		SERUM		LIVER		
	HCO [1]	CO [1]	Cholesterol	Cholic acid	Thiouracil	5 wk.	10 wk.	5 wk.	10 wk.	Total cholesterol	Lipid P	Total lipids	Total cholesterol	Lipid P
	%	%	%	%	%	*gm*	*gm*			*mg %*	*mg %*	%	*mg %*	*mg %*
301	25	–	–	–	–	174 ± 4 [2]	251 ± 7 [2]	0.3	0.8	83 ± 7 [2]	3.9 ± 0.1 [2]	4.1	129	57
302	25	–	1	–	–	157 ± 5	228 ± 11	0.9	1.7	230 ± 19	4.0 ± 1.1	12.3	5340	55
303	25	–	1	1	–	140 ± 4	195 ± 5	0.2	0.1	634 ± 102	7.6 ± 0.7	25.3	12870	49
304	25	–	1	1	0.3	41 ± 2	46 ± 4	0.2	0.5	1980 ± 175	21.1 ± 1.2	20.1	10420	59
305	24	1	–	–	–	218 ± 17	338 ± 11	0	0	92 ± 2	4.1 ± 0.2	4.5	273	59
306	24	1	1	–	–	205 ± 4	328 ± 15	0	0	109 ± 12	4.4 ± 0.2	8.8	1540	55
307	24	1	1	1	–	178 ± 3	291 ± 7	0	0	344 ± 35	6.0 ± 0.5	26.5	13500	55
308	24	1	1	1	0.3	51 ± 2	59 ± 2	0	0	1860 ± 114	23.0 ± 1.7	15.8	6760	62

[1] HCO = hydrogenated coconut oil; CO = corn oil.

[2] Standard error of the mean.

killed at 12 weeks. The dietary additions are shown with the results in table 3.

Growth and dermatosis. The effects on growth and the dermatosis score of adding cholesterol or corn oil to the diet were similar to those previously observed. The addition of 1% of cholic acid to the diet containing 1% of cholesterol further depressed growth. However, at the same time it reduced the scaliness of skin. The rats in this group had developed very little scaliness, even by the end of the experiment. Also, these animals excreted loose feces throughout the experiment and some animals developed diarrhea. The addition of 0.3% of thiouracil together with cholic acid depressed growth severely. In this group, too, cholic acid afforded some protection against scaliness of skin.

Serum constituents. The values reported here for total cholesterol and lipid phosphorus concentrations in serum are the averages for 4 determinations carried out at the end of 5, 8, 12, and 16 weeks. The addition of 1% of cholic acid to the diet containing 1% of cholesterol substantially increased the serum cholesterol level. A tendency for the total cholesterol value to decrease with time was noted in this group. The average value for total serum cholesterol for the group receiving 1% of cholic acid in the diet (634 ± 102 mg %) was nearly three times that for the group supplied 1% of cholesterol only (230 ± 19 mg %). When 0.3% of thiouracil was also fed along with the cholic acid and cholesterol, serum cholesterol concentration was elevated to approximately 2%. Another noteworthy point in this experiment was that the elevation of lipid P showed a rough parallelism with total cholesterol. The addition of 1% of corn oil to the diet again decreased the total amount of cholesterol in the serum of rats receiving 1% of cholesterol alone or with cholic acid, but was ineffective in reducing the concentration of serum cholesterol when 0.3% of thiouracil was also added.

Liver constituents. The values for liver constituents are for animals killed at the end of the experiment (16 weeks).

The livers of the animals in each group were analyzed at the end of 12 weeks but, as the values were similar to those reported for 16 weeks, they are not presented. The addition of 1% of cholic acid to the diet containing 1% of cholesterol greatly increased total lipid and total cholesterol deposition in the liver. Liver size and weight also increased, but when thiouracil was added along with cholic acid and cholesterol, these changes were smaller. This may have been due to the low food consumption of the group receiving thiouracil. The addition of 1% of corn oil was ineffective in reducing total lipid and total cholesterol concentrations in the liver when the diet contained cholic acid and cholesterol. It was effective, however, when the diet contained cholesterol alone.

Experiment 4

It was noted in experiment 3 that the addition of 1% of cholic acid to the diet caused diarrhea and that thiouracil markedly depressed growth; therefore, the addition of thiouracil to the diet was considered inadvisable. Also, it seemed important to study the effects of different levels of cholic acid and the effects of different combinations of cholesterol and cholic acid on serum cholesterol concentration. The results of such a study are recorded in table 4. Six animals were used in each group. Half the animals in each group were killed after blood had been removed at three weeks. The remaining animals were killed after 5 weeks.

Serum cholesterol concentration was affected by both the cholesterol and the cholic acid level of the diet, the latter having the greater influence. The highest values for serum cholesterol concentration were obtained using a diet containing 0.75% of cholesterol and 0.75% of cholic acid; however, this group exhibited some diarrhea. A rough proportionality was noted between serum cholesterol and serum lipid phosphorus concentrations. The highest value for serum cholesterol was associated with the highest for serum lipid phosphorus and *vice versa*. A comparison of these results with

TABLE 4

Effect of various combinations of cholesterol and cholic acid in the diet on serum cholesterol concentration

GROUP NO.	DIET		WEIGHT GAIN		SERUM				LIVER [1]		
					Total cholesterol		Lipid P				
	Cholesterol	Cholic acid	3 wk.	5 wk.	3 wk.	5 wk.	3 wk.	5 wk.	Total lipids	Total cholesterol	Lipid P
	%	%	*gm*	*gm*	*mg %*	*mg %*	*mg %*	*mg %*	%	*mg %*	*mg %*
401	0.19	0.19	82 ± 3 [2]	140 ± 1 [2]	229	221	3.9	3.8	9.4	2550	40
402	0.19	0.38	71 ± 2	121 ± 2	346	213	5.3	4.8	8.6	3280	34
403	0.19	0.75	66 ± 2	101 ± 6	442	342	5.9	5.6	11.5	3870	53
404	0.38	0.19	89 ± 3	145 ± 3	356	281	5.0	4.6	14.8	6210	43
405	0.38	0.38	79 ± 4	119 ± 10	334	399	5.7	5.1	15.6	6830	46
406	0.38	0.75	64 ± 3	109 ± 8	487	455	6.4	6.7	13.6	5930	46
407	0.75	0.19	81 ± 3	122 ± 5	397	338	5.4	5.0	17.8	7680	58
408	0.75	0.38	71 ± 4	112 ± 10	531	444	6.8	6.1	15.7	7370	44
409	0.75	0.75	67 ± 4	108 ± 10	751	590	8.1	6.9	19.7	10170	51
410	1.12	0.38	75 ± 5	121 ± 4	449	431	6.1	5.8	21.7	10750	49

[1] Liver analyses for animals killed after 5 weeks.

[2] Standard error of the mean.

those of the previous experiment indicate that liver cholesterol concentration increased rapidly within the first 5 weeks but that later it changed more slowly. It is also worthy of note that serum cholesterol values were higher at three weeks than at 5 weeks. Hegsted et al. ('57) noted a peak value for serum cholesterol at 4 weeks in rats receiving different combinations of cholesterol and cholic acid in the diet.

DISCUSSION

The main objective of this investigation was to develop a diet suitable for the study of nutritional factors that influence hypercholesteremia in the rat. Therefore, an attempt was made to select a combination of the substances studied which, when added to the diet, would permit a fairly good rate of growth yet would cause a rapid rise in serum cholesterol concentration. In view of the considerations discussed below a diet containing 25% of hydrogenated coconut oil, 1% of cholesterol and 0.5% of cholic acid was selected.

The variations in the fat level of the diet had little effect on serum cholesterol concentration; however, the high level of saturated fat (25% HCO) increased the rate of development of essential fatty acid deficiency as shown by the decreased rate of growth. Deuel et al., ('54) reported that the time required for essential fatty acid depletion is decreased if the concentration of HCO in the diet is increased. Therefore, it was thought that a diet containing the higher fat level would be of advantage in studying effects of essential fatty acids on serum cholesterol levels.

An association between fat level of the diet and serum cholesterol concentration has been reported. Repeated observations in humans (Keys et al., '50; Goren et al., '52; Keys, '56; Anderson et al., '57) have shown that low-fat diets lower serum cholesterol concentration. The absence of such an effect in our experiments was probably due to the rigid exclusion of cholesterol from the diet. The diets used in experiments on man generally contain cholesterol, and a higher fat intake might increase cholesterol absorption.

The observed increase in serum cholesterol concentration upon the addition of corn oil to diets from which cholesterol was excluded, might have arisen from increased endogenous cholesterol synthesis. Avigan and Steinberg ('58) reported higher rates of one-C^{14}-acetate and T_2O incorporation into cholesterol in rats fed corn oil. Corn oil may also increase the transport of cholesterol from other tissues and organs to blood. A rise in blood cholesterol concentration and a lowering in the concentration of cholesterol in the organs was observed by Schettler ('49) in mice fed a diet containing a plant oil but no cholesterol.

Nevertheless, in the present experiments, feeding corn oil along with cholesterol in the diet inhibited the accumulation of cholesterol both in the blood and the liver. A lowering in serum cholesterol concentration as a result of the ingestion of certain vegetable oils has been observed both in humans (Kinsell et al., '53; Bronte-Stewart et al., '56; Beveridge et al., '56, '57) and in experimental animals (Aftergood et al., '57; Bragdon et al., '57; Hegsted et al., '57). This effect of vegetable oils has been attributed to its "solvent action" (Hirsch and Nailer, '56), its essential fatty acid content (Sinclair, '56), its action on the catabolism and excretion of cholesterol (Gordon et al., '57) and its content of a plasma cholesterol depressing factor(s) (Beveridge et al., '57).

Exclusion of pyridoxine from the diet caused some increase in serum cholesterol level; however, the effect was small and it was accompanied by anorexia and severe growth inhibition. Pyridoxine deficiency, therefore, was not considered suitable as a means of producing hypercholesteremia in the rat for routine testing of other dietary constituents. Our results are in agreement with those of Rinehart and Greenberg ('49) who found that the pyridoxine-deficient Rhesus monkey develops hypercholesteremia. Mann and Stare ('55), however, could not confirm their findings. McFarland ('53) observed that high doses of pyridoxine increased atherosclerotic lesions in the chick.

Addition of thiouracil to the diet containing cholesterol and cholic acid caused the greatest increase in serum cholesterol concentration that was observed, but it also caused a severe growth inhibition. Furthermore, the failure of corn oil to reduce the high cholesterol level suggests that this dietary regimen is extremely severe and unsuitable for studying the effects of other dietary components. Hypothyroidism induced by ingestion of thiouracil or I^{131} has been shown to cause hypercholesteremia (Steiner et al., '49; Fillios et al., '56; Page and Brown, '52; Lawrence et al., '58).

The inclusion of 1% of cholesterol in the diet led to an elevation of the serum cholesterol level but since somewhat greater elevation appeared desirable, the addition of cholesterol alone was considered inadequate as a means of producing hypercholesteremia. The inclusion of cholesterol in the diet, however, increased the scaliness of the skin and depressed the growth of essential fatty acid-deficient rats. This observation is in agreement with that of Peifer and Holman ('55) who found that cholesterol enhances essential fatty acid deficiency.

Administration of cholic acid along with cholesterol caused a substantial and rapid increase both in serum and liver cholesterol concentrations; at the same time, it permitted a good rate of growth. With high levels of cholic acid in the diet there was some diarrhea, and with lower levels the serum cholesterol concentration was not elevated appreciably. (Therefore, although the 0.5% level of cholic acid was not tested directly in these experiments, it was selected as being well below the level of 0.75% which caused diarrhea and yet somewhat above the next lowest level which caused an elevation of serum cholesterol without causing diarrhea.) A stimulatory effect of cholic acid in producing hypercholesteremia was demonstrated by Friedman and coworkers ('51, '52) and has been utilized by many workers for the production of high serum cholesterol levels in experimental animals (Page and Brown, '52; Fillios, '57). However, there have been few

studies of the effects of different proportions of cholesterol and cholic acid in the diet on serum cholesterol level. Our results confirm the findings of Hegsted et al., ('57) that the serum cholesterol level is influenced by the levels of both cholic acid and cholesterol in the diet, the former exerting a greater influence.

When cholic acid was fed along with cholesterol in the diet there was a rough parallelism between the concentrations of serum cholesterol and lipid phosphorus. In the absence of cholic acid, serum lipid phosphorus did not show any consistent trend. This suggests that cholic acid might exert its influence by elevating serum phospholipids (Friedman and Byers, '57).

Elevation of serum cholesterol level after different dietary treatments was associated with increased deposition of cholesterol in the liver. Further, the ingestion of corn oil with a hypercholesteremic diet reduced the accumulation of cholesterol both in the serum and the liver. These results suggest that there is a relationship between serum cholesterol concentration and the accumulation of cholesterol in the liver.

SUMMARY

The investigation was undertaken to develop a diet suitable for studying the effects of nutritional factors on cholesteremia in the rat.

A high level of hydrogenated coconut oil in the diet had little effect on serum cholesterol, but it enhanced essential fatty acid deficiency.

Exclusion of pyridoxine from the diet caused a slight elevation of serum cholesterol concentration and a severe growth inhibition.

Feeding of cholesterol led to an appreciable elevation in serum cholesterol concentration and enhancement of essential fatty acid deficiency. The further addition of cholic acid to this diet caused a still greater rise in serum cholesterol concentration. The relative proportions of cholesterol and cholic

acid in the diet determined the serum cholesterol concentration obtained, with cholic acid exerting the greater hypercholesteremic effect. Corn oil added to this diet at a level of 1% reduced the accumulation of serum cholesterol.

There was a considerable elevation of serum cholesterol concentration when thiouracil was also included in the diet, however, thiouracil caused a severe growth depression and the inclusion of 1% of corn oil in the diet containing thiouracil was without effect on the serum cholesterol concentration.

The effects of these dietary changes on serum lipid phosphorus and liver lipid concentrations were also studied.

A diet containing 25% of hydrogenated coconut oil, 1% of cholesterol and 0.5% of cholic acid was selected as suitable for the study of nutritional factors affecting serum cholesterol concentration in the rat.

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STUDIES OF A MANGANESE-IRON ANTAGONISM IN THE NUTRITION OF RABBITS AND BABY PIGS [1,2]

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Previous work by Hartman et al. ('55) showed that 2000 p.p.m. of manganese in the diet interfered with hemoglobin regeneration of lambs. The present investigation was undertaken to study several questions raised by this finding. Specifically, the experiments reported were conducted to obtain information about (a) the effect of excessive manganese in the diet on hemoglobin regeneration of rabbits and baby pigs, (b) the possibility of overcoming the manganese effect by additional iron in the diet, and (c) the minimal level of manganese in the diet that would interfere with hemoglobin regeneration.

EXPERIMENTAL

Four experiments were conducted, one with rabbits and three with baby pigs. The first experiment was designed to determine the effect of excessive manganese on hemoglobin regeneration of rabbits; the second experiment, to determine the effect of excessive manganese and of additional iron in the diet on the hemoglobin regeneration of baby pigs, and the third and 4th, to characterize the minimal level of dietary manganese which would retard hemoglobin regeneration.

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Experiment 1

Procedure. Six Dutch rabbits approximately 6 months of age were fed a diet of dried whole milk for a preliminary period of two months. During the latter part of this period, blood was drawn from the median artery and from ear veins to hasten the onset of anemia. When a pair of rabbits became anemic (5 to 6 gm of hemoglobin per 100 ml of blood), they were assigned at random, one to the control diet and the other to the manganese-supplemented diet (table 1), and were maintained on these diets for 6 weeks. The diets were fed ad libitum. Blood samples (0.02 ml) for hemoglobin determina-

TABLE 1

Diets fed to rabbits in experiment 1

INGREDIENTS	CONTROL	MANGANESE [1]
	gm	*gm*
Chopped soybean hay	1600	1600
Glucose [2]	208	203
Casein (crude)	80	80
Wesson oil	112	112
Manganese mixture [3]	—	5

[1] Supplemented with 2000 p.p.m. of manganese.
[2] "Cerelose," Corn Products Refining Co., New York, N. Y.
[3] Contained 12.3 gm of $MnSO_4 \cdot H_2O$, mixed into 87.7 gm of glucose.

tion were obtained at weekly intervals from ear veins. Hemoglobin was determined by the method of Shenk et al. ('34).

Results. An analysis of the variance of the weekly hemoglobin values over the 6-week period indicated that the hemoglobin regeneration of the control rabbits was significantly greater ($P \leq 0.01$) than that of the rabbits fed 2000 p.p.m. of manganese in the diet. As shown in figure 1, the greatest depressing effect of manganese on hemoglobin regeneration was observed at about the second week; by the 6th week the hemoglobin values of the manganese rabbits approached those of the controls.

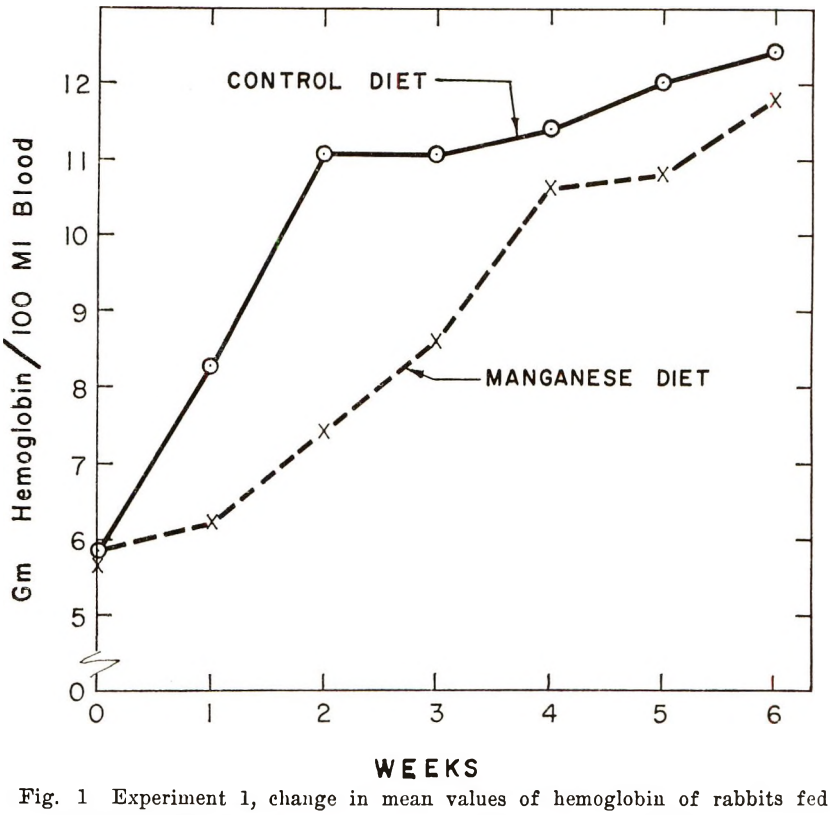

Fig. 1 Experiment 1, change in mean values of hemoglobin of rabbits fed the control and the manganese-supplemented diets during repletion.

Experiment 2

Procedure. Twelve newborn baby pigs deprived of colostrum were administered 10 ml of porcine gamma globulin,[4] 5 ml intraperitoneally and 5 ml subcutaneously, (Barrick et al., '54) and put into individual galvanized wire cages, which were covered with aluminum paint. The basal diet was fresh cow's milk fortified with vitamins A and D. Each pig was restricted to the amount that it would consume 5 times daily. When the pigs became anemic (approximately 5 gm hemoglobin per 100 ml blood), they were stratified into groups of

[4] Porcine gamma globulin was provided by the Armour Laboratories through the courtesy of Dr. M. A. Schooley.

4 according to weight and hemoglobin level and allotted to the following diets in a randomized block design: (1) basal + 25 p.p.m. Fe + 5 p.p.m. Cu; (2) basal + 25 p.p.m. Fe + 5 p.p.m. Cu + 2000 p.p.m. Mn; (3) basal + 400 p.p.m.

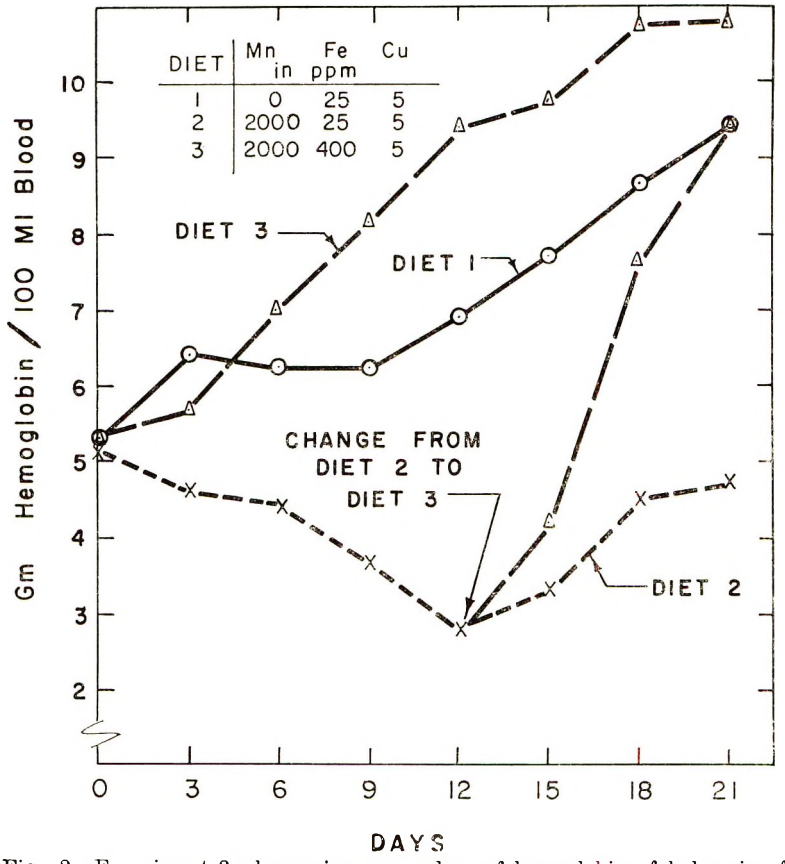

Fig. 2 Experiment 2, change in mean values of hemoglobin of baby pigs fed two levels of iron and two levels of manganese.

Fe + 5 p.p.m. Cu + 2000 p.p.m. Mn. After the pigs were fed these diets 12 days, two of the 4 pigs on diet 2 were changed to diet 3. Hemoglobin measurements were made at three-day intervals throughout the experimental period of 21 days.

Results. As shown in figure 2, hemoglobin regeneration in the baby pig was also depressed by 2000 p.p.m. of manganese

(diet 2). The manganese effect, moreover, was overcome by the addition of 400 p.p.m. of iron (diet 3). The two pigs changed over from diet 2 to diet 3 regenerated hemoglobin at such a rapid rate that by the end of the experiment their hemoglobin values were similar to those fed diets with either 400 p.p.m. of iron or with no supplemental manganese (fig. 2).

Experiment 3

Procedure. The basal diet and general procedure were similar to those of experiment 2. During the experimental period of 27 days, all diets were supplemented with 25 p.p.m. of Fe and 5 p.p.m. of Cu on the dry matter basis. Four levels of manganese supplementation were studied, zero, 125, 250 and 2000 p.p.m. Weights and hemoglobin measurements were taken at 4-day intervals.

TABLE 2

Changes in weight and hemoglobin of baby pigs fed various levels of manganese [1]

DIET NO.	LEVEL OF MN	AV. GAIN PER PIG	AV. HB INCREASE PER 100 ML BLOOD
	p.p.m.	*lbs.*	*gm*
	Experiment 3 [2]		
1	0	28.8	6.09
4	125	26.2	3.38
5	500	26.0	3.18
2	2000	22.7	1.40
L.S.D.[3]		6.04	1.52
	Experiment 4 [4,5]		
1	0	31.3	5.05
6	50	29.3	3.76
7	250	30.9	3.06
8	1250	27.8	3.24
L.S.D.		3.15	1.50

[1] Basal diet was made up of fresh cow's milk plus 25 p.p.m. of Fe and 5 p.p.m. of Cu and vitamins A and D.

[2] Three pigs per diet and an experimental period of 27 days.

[3] Least difference between any two means for significance at 5% level ($P \leq 0.05$).

[4] Six pigs per diet and an experimental period of 25 days for first three replications and 50 days for last three replications.

[5] After 25 days on experiment, iron of pigs on diets of last three replications in experiment 4 was increased from 25 to 50 p.p.m. (see text).

Results. The weight gains and the increase in hemoglobin values made during the experimental period are shown in table 2. The highest average gains were made by the pigs on the zero level of manganese and the lowest by the pigs on the 2000 p.p.m. level of manganese; the difference was significant ($P \leq 0.05$). The increases in hemoglobin levels of the pigs on the 125 and 500 p.p.m. were significantly lower ($P \leq 0.05$) than those on the zero level, and the pigs on the 2000 p.p.m. level made a significantly smaller ($P \leq 0.05$) increment in hemoglobin than did those on either the 125 or 500 p.p.m. levels. The over-all response to manganese appeared to follow a sigmoid curve; e.g., there was a sharp drop in the hemoglobin increment between the first and second levels of manganese, little change between the second and third levels, and a second sharp drop between the third and 4th levels of manganese (table 2).

Experiment 4

Procedure. The basal diet and preliminary period procedure were similar to those in experiments 2 and 3. The levels of manganese studied were zero, 50, 250 and 1250 p.p.m. The pigs were randomized to the diets as in experiment 3. Six replications of the 4 treatments were conducted, three at one time and three at a later time. All 4 diets in the first three replications were supplemented with 25 p.p.m. of Fe and 5 p.p.m. of Cu during an experimental period of 25 days. The last three replications of experiment 4 were carried out in new all-aluminum alloy cages instead of the galvanized wire cages. The 4 diets in the last three replications were supplemented with the usual levels of iron and copper, 25 and 5 p.p.m., respectively, but, since after 25 days the hemoglobin values of none of the pigs on experiment had risen, the iron supplement for all diets was increased from 25 to 50 p.p.m. and the experiment was conducted for an additional 25 days.

Results. The weight gains and the increments in hemoglobin values obtained are shown in table 2. As in experiment

three, the highest gains were made by the pigs on the zero level of manganese and the lowest by those on the highest level, 1250 p.p.m.; the difference was significant ($P \leq 0.05$). The hemoglobin increment made by the pigs receiving the 50 p.p.m. of manganese was not significantly lower than that of those on the zero level, whereas the hemoglobin increments made by the pigs on the 250 and 1250 p.p.m. were significantly lower ($P \leq 0.05$) than of those on the zero level. The shape of the over-all response curve to manganese was exponential rather than sigmoid as in experiment 3.

DISCUSSION

Excess manganese in the diet depressed hemoglobin formation in both mature rabbits and baby pigs. Although manganese retarded hemoglobin formation, regeneration was evident in both species. The hemoglobin regeneration of the rabbits on the high level of manganese was faster than that of the baby pigs, and eventually approached a normal value. This difference in response can be explained, in part at least, by the fact that the anemic baby pigs needed iron not only for hemoglobin repletion but also for hemoglobin increases associated with growth, whereas the mature anemic rabbits needed iron primarily for hemoglobin regeneration. Since the depressing effect of manganese on hemoglobin formation has now been shown to occur with rabbits, baby pigs and lambs (Hartman et al., '55), it appears probable that manganese may have a similar effect on other animal species. The data obtained from baby pigs suggest that the minimal level of manganese which interferes with hemoglobin formation under the experimental conditions prevailing in this investigation lies between 50 and 125 p.p.m. The depressing effect of manganese on growth of pigs was not observed with lambs (Hartman et al., '55). This growth depression was probably the result of the anemia rather than the direct effect of manganese on rate of gain.

The results in experiment 2, showing that additional iron in the diet could overcome the depressing effect of manganese, highlight a similarity between plants and animals in the effect of toxic levels of manganese on the metabolism of iron. The relationship showing that toxic levels of manganese brought about an iron chlorosis in plants has been dealt with by Somers and Shive ('42). They also found that the manganese toxicity of the plant could be overcome by additional iron. According to Somers and Shive ('42), manganese is antagonistic because its oxidizing potential is higher than that of iron. They reasoned that this property of manganese shifted the ionic state of iron to the ferric form which is biologically inactive for plants. This explanation has been challenged by Leeper ('44) and Hewitt ('50). Hartman et al. ('55) suggested that in animals excessive manganese antagonizes the enzyme systems that oxidize or reduce iron at the site of absorption.

The necessity for increasing the supplemental iron from 25 to 50 p.p.m. for the pigs in the last three replications of experiment 4, when the pigs were raised in aluminum cages, suggests that (a) the baby pigs in previous experiments were obtaining some iron from the wire cages, or (b) that 25 p.p.m. of Fe may be an inadequate dietary level for baby pigs. The possibility that the aluminum cages contained a substance which interfered with iron metabolism was deemed unlikely because of the fact that a radioiron (Fe^{59}) study, using two pigs in experiment 4 fed the unsupplemented manganese diet, indicated that 80% or more of the radioiron was absorbed and incorporated into hemoglobin.

SUMMARY

Four experiments, one with mature anemic rabbits and three with anemic baby pigs, were conducted. Excessive manganese in the diet, 2000 p.p.m., depressed hemoglobin formation in both rabbits and baby pigs. The minimal level of manganese in the diet that interfered with hemoglobin forma-

tion was estimated to be between 50 and 125 p.p.m. A supplement of 400 p.p.m. of the iron in the diet overcame the depressing effect of 2000 p.p.m. of manganese on the hemoglobin formation of baby pigs. A supplement of either 1250 or 2000 p.p.m. of manganese in the diet also depressed growth.

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SULFATE METABOLISM IN RABBITS ON HIGH MOLYBDENUM INTAKE[1,2]

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For several years the toxicity to animals of a diet containing a relatively high concentration of molybdenum and a low concentration of copper has been recognized. Dick ('53) noted that increased intake of inorganic sulfate caused a fall in the level of Mo in the blood, accompanied by increased urinary excretion of Mo. Miller and Price ('56) and Miller, Pearson and Denton ('58) reported that the addition of inorganic sulfate to high-Mo diets alleviated the growth-depressing effects of Mo. The present study was carried out to determine what effect a high level of Mo in the diet has on the tissue distribution and excretion of radioactive sulfate that might explain the benefit of added inorganic sulfate. The animal species selected, the rabbit, is one which has been shown to be subject to Mo toxicity effects (Arrington and Davis, '53).

EXPERIMENTAL

Eleven male and 11 female Dutch breed rabbits were weaned at 6 weeks of age and allotted to two groups. One lot (6 males and 5 females) was fed a control diet of rolled oats, alfalfa leaf meal and sucrose, with calcium acetate added to furnish the proper Ca : P ratio. The second lot (5 males and 6 females) received the same basal diet with Mo added as sodium molyb-

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[2] Presented at the 132nd meeting of the American Chemical Society, New York City, September, 1957.

date at a level of 0.15%. After the rabbits had been on the diets for 5 weeks, each received a single oral dose of 10 μc of radioactive sulfur (S^{35}) in the form of sodium sulfate. Following isotope administration, the rabbits were placed in separate cages which allowed individual collection of urine and feces, and were sacrificed 24 hours later. Tissues taken for determination of both radioactive and stable sulfur included liver, muscle, bone and hair. Urine and feces collected were also analyzed. The rabbits consuming the high-Mo diet developed symptoms of Mo toxicity and showed an average weight gain of only 52 gm during the 5-week feeding period, while the control rabbits gained an average of 530 gm each. Consumption of feed by the high-Mo rabbits decreased during the last half of the experimental period.

For the determination of sulfur, tissues were digested in 1% NaOH in a steam bath and aliquots of the digested tissues were dried and ignited in a Parr electric peroxide bomb. Sulfur was then precipitated as $BaSO_4$, filtered onto Whatman No. 42 filter paper on tared Tracerlab discs, dried and weighed to determine total sulfur. The discs then were placed under a thin-end-window Geiger counter for S^{35} measurement. Corrections were made for the absorption of the weak beta emissions of S^{35} by the various amounts of $BaSO_4$ present. The degree of S^{35} activity in the tissue samples was related to the activity determined in a volume of S^{35} solution equal to that administered to each rabbit, and the results expressed as percentage of the administered dose.

RESULTS

In table 1 are presented the findings on the tissue uptake of radioactive S^{35}, expressed in terms of percentage of the dose per gram of tissue, and on excretion of S^{35} in the urine. Significant differences between the two lots were observed only with respect to liver content and urinary excretion.

Differences in total (stable) sulfur concentrations in liver, muscle and hair between control and high-Mo rabbits were not significant, although the concentrations were slightly

higher in the controls. High-Mo livers averaged 1.83 mg sulfur per gram, controls 2.30 mg; high-Mo and control muscle 1.85 and 2.22 mg and high-Mo and control hair, 33.6 and 36.7 mg sulfur per gram, respectively. The low concentration of S^{35} found in the hair was in keeping with the very slow turnover rate recognized in this tissue. Despite a stable sulfur concentration in the hair nearly 20 times as great as that of other tissues, the percentage of the S^{35} dose taken up by the hair in 24 hours was no larger than that taken up by the liver.

One of the more striking effects frequently noted in rabbits subjected to a diet containing Mo at 0.15% or higher is a gross deformity of the humerus. These bones, therefore, were of particular interest in the present study.

TABLE 1

Tissue uptake and excretion of S^{35}

	PERCENTAGE OF DOSE				
	Per gram				Per 24 hr. Urine
	Liver	Muscle	Hair	Humerus	
Control	0.034 [1]	0.007	0.025	0.104	47.7 [2]
High Mo	0.019	0.007	0.018	0.088	54.0

[1] Difference significant at level of 1%.
[2] Difference significant at level of 2.5%.

The bones of the high-Mo rabbits were slightly lighter (average 7.98 gm) than those of the control rabbits (8.75 gm), and the percentage of the S^{35} dose in the entire humerus of each high-Mo rabbit was lower than that of the control humerus, 0.71% compared to 0.88%. The percentage of the dose per gram of humerus was also lower in the high-Mo rabbits, although in neither case was the difference significant. With respect to total sulfur, humeri from the control rabbits contained the smaller amount, averaging 28.8 mg of sulfur, or 3.2 mg of sulfur per gram of fresh bone, while those of the high-Mo rabbits averaged 40.1 mg of sulfur per bone or 5.01 mg per gram of bone. The difference between lots was significant at the 0.5% level.

Urinary excretion of S^{35} in 24 hours was significantly ($P < 0.025$) higher in the high-Mo than in the control rabbits, with 54.0% of the dose excreted in the urine by the high-Mo rabbits against 47.7% in the controls. Urinary total sulfur excretion for 24 hours, averaging 93.7 mg for each control rabbit compared to 107.3 mg for each high-Mo rabbit, did not differ significantly in the two lots of rabbits. The findings with respect to amounts of S^{35} and total sulfur excreted via the feces were too erratic to be of any value, probably due to coprophagy.

In view of the fact that sulfur usually serves as an indicator of protein metabolism, it was believed that the excretion of a higher amount of urinary sulfur by the high-Mo rabbits might indicate that these rabbits had also excreted more nitrogen than the controls. Analysis of the urines for nitrogen revealed an average excretion of 27 mg per control rabbit, compared with 63 mg for the high-Mo rabbits, the difference being significant at the 5% level. If the control rabbits are considered to be in nitrogen balance or even slightly on the positive side, since they were in the growing stage, then the high-Mo rabbits were probably in negative nitrogen balance. Since the rabbits on high-Mo intake showed weight losses during the last two weeks on the diet, this finding is not surprising. In wasting due to starvation, however, investigators have found that the urinary N/S ratio remains virtually unchanged from the N/S ratio observed in animals on feed. In the present study the N/S ratio of the high-Mo rabbits was twice as high as that of the controls, indicating definite toxic effects of the high intake of Mo over and above those due to decreased food intake.

DISCUSSION

Studies carried out with rats by Miller and Price ('56) and with chicks by Miller, Pearson and Denton ('58) and by Reid, Davies and Couch ('58) showed that the growth-retarding effects of a high intake of Mo could be alleviated by increasing the inorganic sulfate content of the diet. The results of the present study indicate that an important factor in the beneficial effect of sulfate lies simply in its replacement value.

Tissue retention of the ingested radiosulfate was depressed by the high-Mo diet. Urinary excretion values for the radiosulfate, higher in the high-Mo rabbits, contraindicate any failure in the intestinal absorption of sulfate due to Mo.

In regard to the skeletal (humeral) deformity frequently observed in molybdenosis in certain animal species, the humeri of the high-Mo rabbits, although lighter in weight, were found to contain more total sulfur than those of the control animals. Most of the sulfur present in the skeleton occurs in the cartilage as chondroitin sulfate; the increased sulfur content observed may be due therefore to a slowdown in the process of ossification, resulting in the presence of a higher proportion of cartilaginous tissue in these rabbits which were in the growing stage.

SUMMARY

Rabbits fed 0.15% of molybdenum in the diet for 5 weeks developed Mo toxicity symptoms and were given oral doses of radioactive S^{35}. Levels of S^{35} found in the livers and bones of these rabbits were lower than in rabbits maintained on a control diet, and urinary excretion of S^{35} higher, indicating decreased retention of sulfur in the high-Mo animals. Part of the benefit of added sulfate in high-Mo diets may lie in its replacement action. Total sulfur content of the humeri of the high-Mo rabbits was significantly higher than in control rabbits, indicating a higher proportion of cartilage and a possible retarding effect of Mo on the process of ossification.

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EFFECT OF HIGH MOLYBDENUM INTAKE ON THE DISTRIBUTION AND EXCRETION OF CA^{45} AND P^{32} IN THE RABBIT [1]

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The toxic effect on cattle of a high dietary level of molybdenum was established by Ferguson and co-workers ('43). Arrington and Davis ('53) noted that rabbits fed a diet containing Mo at a level of 0.2% showed symptoms somewhat similar to those seen in cattle, including deformities of the joints of the extremities, anemia and diarrhea. Reid et al. ('58) observed that purified diets containing 0.05, 0.2 and 0.6% Mo resulted in, respectively, 10, 50 and 80% reductions in the growth of chicks. The bone deformity seen in rabbits was strongly suggestive of a disturbance in calcium and phosphorus metabolism and the present study was undertaken to determine, through the use of the radioactive forms of Ca and P, if such a disturbance does occur in rabbits subjected to a diet containing a high level of Mo.

EXPERIMENTAL

Thirty female Dutch breed rabbits 5 weeks of age were allotted to two groups. One lot was fed a control diet of rolled oats (88%), alfalfa leaf meal (10%) and sucrose (2%) with calcium acetate added to furnish the proper Ca: P ratio. The second lot received the same basal diet with Mo added at a level of 0.2% as sodium molybdate. After the rabbits had been

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fed the diets for 5 weeks, each received a single oral dose containing 3 μc each of Ca^{45} and P^{32} and was placed in a cage equipped to provide separate collection of urine and feces. Three rabbits from each lot were sacrificed after the following time intervals: 6 hours, 24 hours, 48 hours, 7 days and 14 days. Tissues taken for determination of both radioactive and stable Ca and P included blood, liver, kidney, skeletal muscle, cardiac muscle and bone. Urine and feces collected at 24, 48, 96 and 168 hours after dosing were also analyzed for both the radioactive and the stable isotopes of Ca and P.

RESULTS

Feed consumption by the rabbits receiving the ration containing added Mo was slightly lower than by the control rabbits. This factor was partially responsible for the difference in weights of the rabbits at sacrifice, the control animals averaging 2014 gm as compared with 1701 gm for the high-Mo animals. However, the appearance of the high-Mo rabbits indicated effects far beyond those which would be expected from a slight decrease in food intake, as all the symptoms of Mo toxicity mentioned above were present.

Chemical and radioisotopic analyses of the soft tissues of the rabbits in the two dietary groups revealed no appreciable effect of the level of Mo in the diet on uptake of radioactive Ca or P, or on tissue levels of total Ca or P, except in the heart and liver. Ca^{45} concentrations were slightly, though not significantly, higher in the hearts of the high-Mo rabbits than in the controls. Both P^{32} and Ca^{45} concentrations were slightly higher in the livers of the high-Mo rabbits.

The amounts of Ca^{45} and P^{32} excreted by the control rabbits exceeded the amounts excreted by the high-Mo rabbits only slightly, except in the fecal excretion of P^{32}. The control animals excreted significantly more ($P < 0.05$) of this isotope via the intestine during the 7-day collection period (54% of the amount administered) than did the high-Mo rabbits (47%). Rabbits of the control group also excreted more total Ca and P in both urine and feces than the high-Mo rabbits, due to higher feed consumption by the control animals.

TABLE 1

Soft tissue levels and excretion of radioactive and stable Ca and P in the rabbit following oral administration of Ca^{45} and P^{32}

HR. AFTER DOSING	% DOSE/GM 10^3					HR. AFTER DOSING	% DOSE/RABBIT	
	Blood	Liver	Kidney	Skel. muscle	Heart		Urine	Feces
				Calcium ^{45}				
6	10.7	6.7	3.4	8.2	3.5	0–24	7.7	26.4
24	1.3	0.5	1.5	0.3	0.9	24–48	2.1	5.0
48	0.4	0.5	0.7	0.3	0.2	48–96	1.0	1.1
168	0.2	0.1	0.0	0.0	0.0	96–168	0.5	0.3
336	0.1	0.0	0.0	0.0	0.0			
Total Ca, mg/gm gm tissue	0.05	0.05	0.12	0.03	0.12	mg/day	20.8	104
				Phosphorus ^{32}				
6	100	35	55	55	5	0–24	0.8	35.7
24	60	57	48	31	8	24–48	0.3	11.7
48	40	55	43	26	10	48–96	0.3	5.1
168	5.1	33	24	15	12	96–168	0.3	1.5
336	3.2	28	17	12	12			
Total P, mg/gm tissue	0.47	3.54	2.83	2.18	2.06	mg/day	5.3	212

Although the findings obtained in this study with respect to the tissue distribution and turnover of orally administered Ca^{45} and P^{32} and of total Ca and P in the soft tissues failed to demonstrate any appreciable effects of the high Mo intake, a summary of the combined average values for both lots of rabbits is presented in table 1. These data are considered indicative of normal Ca and P metabolism in the rabbit and are presented because the authors have found no similar published information. A high intake of Mo did affect the excretion of Ca and P, and in order to adhere to the scheme of normal values, averages for the control lot only are shown for urine and feces in this table.

TABLE 2

Percent of administered Ca^{45} and P^{32} per gram of humerus

TREATMENT	HR. AFTER DOSING				
	6	24	48	168	336
			Calcium ^{45}		
Control	0.13 ± 0.04 [1]	0.29 ± 0.05	0.29 ± 0.04	0.27 ± 0.03	0.18 ± 0.01
High Mo	0.19 ± 0.06	0.33 ± 0.03	0.36 ± 0.11	0.30 ± 0.05	0.44 ± 0.09
			Phosphorus ^{32}		
Control	0.10 ± 0.06	0.20 ± 0.04	0.16 ± 0.03	0.23 ± 0.05	0.23 ± 0.04
High Mo	0.08 ± 0.04	0.17 ± 0.04	0.21 ± 0.05	0.23 ± 0.01	0.40 ± 0.04

[1] Standard deviation.

In view of the skeletal deformities observed in the rabbits on high Mo intake, the tissue which was expected to show the greatest alterations in Ca and P content was bone, in which decreased contents of stable Ca and P and decreased uptake of Ca^{45} and P^{32} were anticipated. Results of analyses of the humeri of the rabbits did not bear out this prediction. Ca^{45} and P^{32} findings, presented in table 2, show that the uptake of Ca^{45} was slightly greater in the humeri of the high-Mo rabbits than in the controls, and apparently continued over a longer period of time. The level of Ca^{45} in these bones was higher 14 days (336 hours) after dosing than at 7 days, while control levels were lower. While the uptake of P^{32} was somewhat less rapid in the humeri of the high-Mo rabbits during

the first 6 hours, P^{32} levels in these animals exceeded control levels between 24 and 48 hours after isotope administration, and at 14 days were nearly twice as high as control P^{32} values.

The absolute amounts, as well as the concentrations, of Ca^{45} and P^{32} were higher in the humeri of the high-Mo rabbits than in the controls, even though the 14-day bone weights were lower in the former group. Thus, rather than a decrease in the rate of bone Ca and P metabolism with high Mo intake, an increased uptake of these elements was found to have occurred.

The findings with respect to total Ca and P in the humeri are presented in table 3. Weights were obtained with the bones in the fresh state.

TABLE 3

Average total Ca and P content of whole humeri

HR. AFTER DOSING	24		48		168		336	
	Ca	P	Ca	P	Ca	P	Ca	P
	mg		*mg*		*mg*		*mg*	
Control	607	272	576	286	559	267	694	314
	(4.44)[1]		(4.23)		(4.26)		(5.48)	
High Mo	568	260	532	265	590	267	501	250
	(4.40)		(4.24)		(4.43)		(2.92)	

[1] Av. wt. of humeri, gm.

Total Ca content of the high-Mo rabbit humeri decreased during the second week after isotope administration (7th week of high Mo intake) from 590 to 501 mg of Ca, while Ca in control rabbit humeri increased from 559 to 694 mg. During this period the P content decreased from 267 to 250 mg per humerus in the high-Mo rabbits, controls showing an increase from 267 to 314 mg. These reductions of 15% in Ca and 6% in P in the high-Mo humeri are surprisingly small in the face of the 34% reduction in bone weight during the same period. In the rabbits sacrificed at 14 days, high-Mo humeri contained 172 mg Ca per gram against 127 mg per gram in the control humeri. The values for P were 86 and 57 mg per gram for humeri of the control and high-Mo rabbits, respectively.

DISCUSSION

The alteration in bone structure accompanying molybdenosis was reported by Comar et al. ('49), who noted that weanling rats fed high levels of Mo in the diet showed retarded skeletal growth, and by Arrington and Davis ('53), Lindenstruth ('54), and Roberts ('56). The investigators carrying out the present study suspected that the skeletal disorders might indicate a failure in absorption of Ca or P or both due to formation in the intestine of insoluble salts of these cations with the molybdate radical. The data obtained in this study, however, indicate that absorption of Ca and P took place at a normal rate in the rabbits ingesting large amounts of Mo. If absorption of these elements from the intestine had been impaired, fecal excretion of Ca^{45} and P^{32} would have been appreciably higher in the high-Mo rabbits, and urinary excretion lower, following oral administration of these radioisotopes. This was not the case. Further, soft tissue levels of Ca^{45} and P^{32} were as high in the high-Mo rabbits as in the control animals, indicating unimpaired absorption.

Weights of humeri from the high-Mo animals sacrificed two weeks after isotope administration (after 7 weeks on the diet) averaged 2.92 gm, as compared with 5.48 gm in control animals sacrificed at the same time. The total amounts of Ca and P present in the smaller bones from the high-Mo rabbits were nearly as high as those in the control bones, and the amounts of radioactive Ca and P were higher than in the controls. It is presumed, therefore, that the decrease in weight of the humeri was the result of a diminution in the amount of organic matter. If part of the tissue lost is represented by marrow, this might explain the anemia commonly observed in Mo toxicity. Roberts ('56) made histological studies of bones from the extremities of rabbits on high-Mo intake and reported areas of necrosis in the marrow and a decrease in the number of nucleated red blood cells in the blood sinuses. He noted that there was no evidence of defective deposition of inorganic materials in the cartilage matrix.

It is possible that Mo exerts its toxic action through an effect on certain enzyme systems. Bossard ('47) observed that sodium molybdate inhibited all phosphatases found in extracts of plant tissues. Richert and Westerfeld ('53) isolated from soy flour a Mo salt essential to the deposition and maintenance of normal levels of rat intestinal xanthine oxidase. It would appear that Mo, up to a certain concentration, is necessary to the action of certain enzymes, but beyond this level acts as an inhibitor.

SUMMARY

Rabbits fed a diet containing 0.2% of Mo developed symptoms of Mo toxicity and were given oral doses of Ca^{45} and P^{32}. Neither soft tissue levels nor urinary or fecal excretion of total or radioactive Ca or P indicated any impairment in the absorption of these ions from the intestine. Bone disorders observed in the rabbits on high-Mo intake were not due to a failure in deposition of Ca or P, and are presumed to result from a failure in the organic phase of bone metabolism.

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THE EFFECT OF ANTIOXIDANTS ON VITAMIN E DEFICIENCY SYMPTOMS AND PRODUCTION OF LIVER "PEROXIDE" IN THE CHICKEN

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Non-tocopherol antioxidants are known to prevent vitamin E deficiency symptoms in animals (Dam, '57). Singsen et al. ('55) proposed that the antioxidant DPPD (diphenyl-*p*-phenylenediamine) functions in a manner parallel to vitamin E. This view has not been accepted by many workers. Several groups (Ames, '56, Christensen et al., '56) concluded that antioxidants are effective because they prevent the destruction of vitamin E by oxidizing unsaturated fatty acids in the diet. Shull et al. ('57) suggest that in the guinea pig, antioxidants protect against muscular dystrophy by conserving tissue tocopherols.

The studies presented here demonstrate that the antioxidant Santoquin [1] prevents vitamin E deficiency symptoms by its effect in the tissues of the chicken. Since the diets used contained negligible amounts of vitamin E, it was concluded that either this antioxidant can function in a manner parallel to vitamin E or that the specific requirement for vitamin E is extremely small. It was also found that the fatty acids in the livers of vitamin E-deficient chickens are very susceptible to peroxidation.

[1] Registered trademark of Monsanto Chemical Company for 1,2-dihydro-6-ethoxy, 2, 2, 4-trimethylquinoline.

EXPERIMENTAL

In each experiment, day-old crossbred cockerels from a commercial hatchery were fed the basal diet for one week. They were then weighed, divided into groups of approximately equal weight distribution and fed the experimental diets for three weeks. The birds were reared under electrically heated brooders in wire-floor cages in an air-conditioned room. The basal diets are given in table 1.

TABLE 1

Vitamin E-deficient purified diets

INGREDIENTS	DIET NUMBER		
	EC15G20	EC20G15	EMS35
	%	%	%
Casein (purified)	15.0	20.0	——
Gelatin	10.0	15.0	——
Assay Protein C-1 [1]	——	——	35.0
L-Arginine HCl	0.6	——[2]	——
Stripped lard [3]	4.0 [4]	16.0 [4]	16.0
Salts [5]	6.0	6.0	6.0
Glycine	——	——	1.0
Ground cellulose (Alphacel)	——	——	1.0
Vitamin mixture [6]	0.5	0.5	0.5
Choline chloride	0.2	0.2	0.2
Vitamin A concentrate (20,000 I.U./gm)	0.04	0.04	0.04
Vitamin D concentrate (7500 I.C.U./gm)	0.011	0.04	0.03
Procaine penicillin G	0.001	0.001	0.001
Cerelose (glucose)	63.65	44.22	40.23
Total	100.00	100.00	100.00
Maximum vitamin E content (I.U./lb)	0.09	0.36	0.36

[1] An isolated soybean protein obtained from Archer- Daniels-Midland Company, Cincinnati, Ohio.

[2] In experiment 2, 0.6% of L-arginine·HCl was added.

[3] Obtained from Distillation Products Company, Rochester 3, N. Y. This product contains less than 5 μg of tocopherols per gram of fat and is assumed to be the principal source of vitamin E in these diets.

[4] 0.1% Tenox VI added. This product contains 10% BHT and 10% BHA and can be obtained from Eastman Chemical Products, Inc., Kingsport, Tennessee.

[5] Contains in grams per 60 gm the following compounds: $MgCl_2$, 4.1; $CaCO_3$, 15; K_2HPO_4, 9; $Ca_3(PO_4)_2$, 14; NaCl, 8.8; Na_2HPO_4, 7.3; ferric citrate, 0.4; $MnCl_2$, 0.37; KI, 0.04, $ZnCO_3$, 0.02; $CuCl_2$, 0.03.

[6] This will supply in milligrams per kilogram of finished diet: vitamin B_{12}, 0.03; biotin, 0.30; menadione, 1.0; pyridoxine·HCl, 8.0; folic acid, 4.0; riboflavin, 16.0; calcium pantothenate, 20.0; thiamine·HCl, 24.0; nicotinic acid, 100.0. This mixture, Alphacel, and salts can all be obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio.

The formation of oxidized unsaturated fatty acids (hereinafter referred to as "peroxide") in the liver was measured by a modification of the method of Bernheim et al. ('57). This consisted of homogenizing 0.5 to 1.0 gm of liver in sufficient M/15 Na-K phosphate buffer at pH 7.0 to have a final concentration of 20 mg liver (wet wt.) per ml. Four milliliters of this homogenate were shaken in air at 37°C in a 25-ml Erlenmeyer flask. After one hour, 2 ml of 20% trichloroacetic acid were added. The precipitate was removed by centrifugation and 2.0 ml of 0.67% thiobarbituric acid (TBA) were added to the clear supernatant. The mixture was placed in a boiling-water bath for 10 minutes. The tubes were cooled and the optical density of the final solution was measured at 530 mμ in a colorimeter. The results are the average of 6 individual determinations.

Dietary "peroxide" was determined as follows: after standing at room temperature for 4 weeks, a 20 gm sample of each diet was placed in a 50-ml glass-stoppered Erlenmeyer flask. Twenty-five milliliters of reagent grade benzene were added and nitrogen bubbled through the suspension for 30 seconds. The flask was then stoppered, shaken for two minutes, allowed to stand for 5 minutes and filtered. The "peroxide" determination was then carried out as described by Sidwell et al. ('54).

RESULTS

Experiment 1. In the first experiment (table 2) three different purified diets were used. The resulting pattern of vitamin E deficiency symptoms was different in each case. With a diet containing 4% of "stripped" lard the main symptom was muscular degeneration. High mortality observed in chicks receiving the basal diet was evidently a result of excessive destruction of dietary vitamin A. Santoquin, BHT (2,6, ditertiary-butyl-*p*-cresol), and MHA [2] (methionine hydroxy analogue) all tended to preserve vitamin A. In con-

[2] Registered trademark of Monsanto Chemical Company for calcium DL-2-hydroxy-4-methyl thiobutyrate.

trast to what would be expected from a source of free radicals, lauroyl peroxide also tended to act as an antioxidant. Either 0.60% of MHA or 0.10% of Santoquin in the diet resulted in complete prevention of muscular degeneration. This extends findings previously reported by Machlin and Shalkop ('56).

When diets containing higher levels of protein and 16% of "stripped lard" were used, either encephalomalacia (diet EC20G15) or all three symptoms of vitamin E deficiency appeared (diet EMS35).

TABLE 2

Experiment 1, vitamin E deficiency symptoms with purified diets

SUPPLEMENTS	SURVIVORS (27 DAYS)	VITAMIN A [1] 1000 I.U./KILO	SYMPTOMS [2]
None (EC15G10)	3/36	2	None (dead)
+ 0.60% MHA [3]	22/24	9	None
+ 0.01% Santoquin	23/24	18	10/12 M.D.
+ 0.10% Santoquin	22/24	27	None
+ 0.10% BHT [4]	22/24	17	9/11 M.D.
+ 0.50% lauroyl peroxide	32/36	4	10/10 M.D.
None (EMS35)	15/24	17	2/13 M.D. 5/13 E.D. 6/24 Enc.
None (EC20G15)	7/24	18	15/24 Enc.

[1] Analyzed at end of experiment.

[2] M.D. = Muscular degeneration (white striations); E.D. = Exudative diathesis; Enc. = Encephalomalacia. Numerator is the number of birds with obvious symptoms; denominator is the number of birds examined.

[3] Registered trademark of the Monsanto Chemical Company for calcium DL-2-hydroxy-4-methylthiobutyrate.

[4] 2,6, Ditertiary-butyl-*p*-cresol.

Experiment 2. In view of the report of a high arginine requirement with casein diets (Hogan et al., '57) arginine was added to the 20% casein, 15% gelatin diet (EC20G15). With MHA in the diet (table 3) 100% of the birds showed signs of encephalomalacia within 18 days. The addition of 0.02% of Santoquin prevented encephalomalacia but had no effect on the muscular degeneration observed in birds surviving to 24 days. Lauroyl peroxide reduced the incidence of encephalomalacia. A supplement of 0.004% *dl*, α-tocopherol acetate prevented both symptoms.

TABLE 3

Effect of Santoquin, BHT,[1] and MHA[2] on vitamin E deficiency systems and on liver and dietary "peroxide"

DIET AND SUPPLEMENTS	GAIN	SYMPTOMS		TBA INDEX (OPTICAL DENSITY)	
				Liver Assay	Diet
Exp. 2	*7–18 days*	*18 days*	*24 days*		
None (EC20G15)[3]	158	8/14 Enc.	——	0.17 ± 0.021	—[4]
+ 0.02% Santoquin	173	0/14 Enc.	5/5 M.D.	0.13 ± 0.018	
+ 0.02% BHT	174	5/14 Enc.	4/5 M.D.	——	—
+ 0.60% MHA	178	14/14 Enc.	——	——	—
+ 1.0% lauroyl peroxide	114	2/14 Enc.	2/6 M.D.	——	—
+ 0.004% *dl*, α-tocopherol acetate	180	0/14 Enc.	0/5 M.D.	0.01 ± 0.004[5]	—
Exp. 3	*6–24 days*	*24 days*			
None (EMS35)	101	41/45 Enc.		0.18 ± 0.055	2.7
+ 0.02% Santoquin	139	0/15 Enc.		0.09 ± 0.047	0.1
+ 0.70% MHA	167	11/15 Enc.		0.24 ± 0.088	1.5
+ 0.70% MHA + 0.02% Santoquin	176	0/15 Enc.		0.22 ± 0.092	0.1
+ 0.70% MHA + 0.10% Santoquin	189	0/15 Enc.		0.03 ± 0.003	0.0
Exp. 4	*6–18 days*	*18 days*	*24 days*		
None (ES35)[6]	144	5/14 E.D.	5/13 M.D.	——	1.8
+ 0.002% Santoquin	179	5/15 E.D.	7/13 M.D.	——	0.0
+ 0.010% Santoquin	201	4/12 E.D.	7/12 M.D.	——	0.0
+ 0.050% Santoquin	201	0/13 E.D.	0/13 M.D.	——	0.0

[1] BHT = 2,6, ditertiary-butyl-*p*-cresol.

[2] MHA = methionine hydroxy analogue.

[3] 0.6% L-arginine·HCl added.

[4] Not determined.

[5] ± standard deviation.

[6] Diet EMS35 plus 0.7% MHA.

Appreciable amounts of "peroxide" were produced in livers of chicks fed the basal diet. Dietary vitamin E completely prevented "peroxide" formation whereas 0.02% of Santoquin had only as light effect.

TABLE 4

In vitro liver peroxide study [1]

COMPOUND	AMOUNT REQUIRED TO REDUCE TBA VALUE 50%	
	(Micrograms/tube)	P.P.M. in liver
Santoquin	0.2	2.5
dl, α-tocopherol	1.5	18.7
BHT [2]	2.0	25.0
2-methyl 1,4,-napthoquinone bisulfite [3]	15.0	187.5
Serotonin creatine sulfate	125.0	156.0
Selenium (as Na_2SeO_3 $5H_2O$)	over 200.0	over 2500.0

[1] All compounds were added to the homogenates before incubation. Santoquin, *dl*, α-tocopherol and BHT were dissolved in olive oil. A 1% emulsion of the olive oil solutions in pH 7.0 buffer was prepared after addition of 0.25% Tween 80. The olive oil and Tween 80 combination alone had no effect on liver peroxide.

[2] BHT = 2,6, ditertiary-butyl-*p*-cresol.

[3] As Klotogen F (Abbott Laboratories, Chicago).

Experiment 3. In this experiment C-1 assay protein [3] was used (diet EMS35). A supplement of 0.02% of Santoquin was able to prevent oxidative rancidity in the diet as measured by the TBA assay but did not completely prevent liver "peroxide" formation. When 0.10% of Santoquin was added however, liver "peroxide" was reduced to an almost negligible quantity. The observation that Santoquin is effective in reducing liver "peroxide" formation was confirmed with *in vitro* studies (table 4). In the *in vitro* study, Santoquin was more than 8 times as effective as *dl*, α-tocopherol.

Experiment 4. In this experiment low levels of Santoquin (0.002, 0.010 and 0.050%) were tested. They were found to suffice in preventing oxidative rancidity in the diet (ES35) but not for the prevention of vitamin E deficiency symptoms (table 3).

[3] Archer-Daniels-Midland Company, Cincinnati, Ohio.

DISCUSSION

Santoquin is evidently more effective than DPPD in the prevention of muscular degeneration since only 0.10% was sufficient to completely prevent this symptom, in contrast to the requirement of 0.15 to 0.25% for DPPD, found in an earlier study (Machlin and Shalkop, '56). As with DPPD the level necessary to prevent muscular degeneration is much higher than the level required to prevent encephalomalacia (0.02% in this study).

The beneficial effect of lauroyl peroxide tends to parallel the report of Witten and Holman ('52) who found that either vitamin E or benzoyl peroxide stimulated the growth of fatty acid-deficient rats when fed with linoleic acid. Peroxides decompose to free radicals (Bolland and Gee, '46) which initiate the oxidation of unsaturated fatty acids. Since fatty acid oxidation will aggravate a vitamin E deficiency, the beneficial effects of such compounds are unexpected. Perhaps vitamin E sometimes functions as a pro-oxidant in the body and the peroxides simulate this activity. At high concentrations tocopherol has been reported to act as a pro-oxidant in the oxidation of unsaturated fatty acids (Quackenbush, '49).

A deficiency of antioxidants in the livers of vitamin E-deficient chicks was made apparent by the production of liver "peroxide" as estimated by the TBA method. The TBA method is not specific but is believed to be chiefly a reflection of the formation of oxidized linolenic acid (Wilbur et al., '49). A dietary supplement of 0.004% of vitamin E (*dl*, α-tocopherol acetate) or 0.100% of Santoquin almost completely prevented liver "peroxide" formation. When added directly to the liver homogenates, Santoquin was even more effective than vitamin E (*dl*, α-tocopherol) in inhibiting "peroxide" formation. Evidently liver "peroxide" formation is a reflection of an antioxidant deficiency, not specifically a lack of vitamin E. In these studies there was no evidence for the presence of "peroxide" in the liver before incubation of the homogenate. However, using a somewhat more sensitive procedure, Tappel and Zalkin ('58) did detect significantly more "peroxide" in

the livers of vitamin E-deficient rabbits than in control vitamin E-replete animals, thus demonstrating that *in vivo* peroxidation of liver lipids can occur. *In vivo* peroxidation of fatty acids in the liver could easily account for the observation that vitamin A is destroyed in the tissues of vitamin E-deficient animals (Hickman et al., '44; Sherman, '41; Dam et al., '52), since carotene (and presumably vitamin A) destruction is known to be correlated with peroxidation of fatty acids (Hove and Hove, '44). Both Hove ('55) and Tappel and Zalkin ('58) have suggested that inhibition of sensitive mitochondrial enzymes by lipid peroxidation might account for the manifestation of vitamin E deficiency symptoms. In the present study, lipid "peroxide" formation occurred in livers of birds showing no signs of encephalomalacia (table 3). However, prevention of muscular degeneration did seem to be correlated with prevention of liver "peroxide" formation. It is likely therefore, that the injurious effects of lipid peroxidation cannot account for all of the manifestations of a vitamin E deficiency.

The purified diets used in these experiments contained less than 0.09 I.U. of vitamin E per lb. (4% lard diets) or 0.36 I.U. vitamin E per lb. (16% lard diets). It is therefore apparent that antioxidants can prevent vitamin E deficiency symptoms when added to diets containing very low dietary levels of this vitamin. Since vitamin E deficiency symptoms occurred in chicks fed diets containing fats that did not oxidize (table 3) and presumably therefore did not destroy the low levels of vitamin E present, the higher levels of Santoquin which did prevent vitamin E deficiency symptoms must have had their effect in the body of the chicken. The antioxidant could prevent vitamin E deficiency symptoms in two ways, one by protecting the body stores of vitamin E "carried over" in the egg or secondly, by replacing vitamin E in some biological function(s). The finding that the enzyme cytochrome-c reductase contains alpha-tocopherol and that other antioxidants cannot replace the vitamin in this system (Nason et al., '57) suggests that there is a specific biochemical requirement for

alpha tocopherol. However, based on the present studies, the specific dietary requirement for vitamin E, even in diets containing large amounts of unsaturated fatty acids must be extremely low. Moreover, in the chicken at least, antioxidants have been able to function in the same way as vitamin E in all experiments in which torula yeast was not used when an appropriate antioxidant was tested at dietary levels over 0.05%. It can be concluded that in the chicken, as was reported for the rat (Draper et al., '58), the requirement for vitamin E "is largely represented by a need for a biologically active antioxidant."

CONCLUSIONS

1. A purified diet was developed which results in 100% incidence of encephalomalacia in less than three weeks when fed to chicks from dams not depleted of vitamin E.

2. Additions of 0.10% of Santoquin (1,2-dihydro-2,2,4-trimethyl-6-ethoxy-quinoline) completely prevented muscular degeneration in chicks fed a diet containing less than 0.09 I.U. vitamin E per pound.

3. Vitamin E deficiency symptoms were produced in chicks receiving diets in which there was no detectable oxidative rancidity of dietary fat.

4. Santoquin was able to prevent encephalomalacia and exudative diathesis when added to diets containing less than 0.36 I.U. vitamin E per lb.

5. Using a thiobarbituric acid colorimetric procedure, "peroxide" formation was observed in liver homogenates of chicks fed vitamin E-deficient diets. Addition of vitamin E or Santoquin to the diet or directly to the homogenates prevented the liver "peroxide" formation.

6. It was concluded that the major portion of the requirement of the chicken for vitamin E can be satisfied by a biologically active antioxidant.

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