STUDIES ON CHRONIC VITAMIN B_6 DEFICIENCY IN THE RAT

I. CHANGES IN THE INTACT ANIMAL

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ONE FIGURE

The role of the vitamins of the B_6 group has been firmly established in certain metabolic reactions (Snell, '53). The effects of a deficiency of this group have been investigated in different species and strains and apparent discrepancies have been noted (Agnew, '51). While growth effects and xanthurenic acid excretion have been established for several species (Anonymous, '47), the hematologic picture has been a point of controversy (Agnew, '49). In addition, histologic findings in the rat have not been well defined (Calder, '42, '44). In the present investigation the effects of a chronic vitamin B_6 deficiency in the albino rat were studied in respect to growth, hematologic and histologic changes, blood pressure, extra-cellular space, and xanthurenic acid excretion. Further studies on organ weights, vitamin B_6 content of tissues and oxygen consumption of tissues will be presented in another paper (Olsen and Martindale, '54).

EXPERIMENTAL

Weanling, male albino rats of the Wistar strain were placed on a complete, purified diet for a period of 4 weeks to obtain basal growth and blood pressure levels. Blood pressure was

measured by the foot-cuff method (Kersten et al., '47). At the end of the conditioning period the rats were divided into groups receiving the following dietary regimens ad libitum: (1) complete purified diet — Casein (Labco) 24%, dextrose 62%, hydrogenated vegetable oil 10%, salt mixture *W* 4%, and vitamin mixture [1] — (control — 35 animals); (2) Purina laboratory checkers (diet control — 15 animals); (3) complete purified diet with the pyridoxal hydrochloride replaced by its antimetabolite, desoxypyridoxine, at a level of 1.25 mg for 100 gm of diet (deficient — 100 animals). A 4th group received the complete purified diet *with restricted intake* to serve as weight controls for the B_6 deficient animals (restricted controls — 20 animals).

After 4 weeks the deficient group was further subdivided: A small number of animals were continued on the diet containing desoxypyridoxine and the majority of the group was kept on a B_6 deficient diet with no pyridoxal or desoxypyridoxine. At the end of a total of 30 weeks from the conditioning period the experimental regimen was altered: (1) the deficient animals were given either desoxypyridoxine (1.25 mg /100 gm diet) or pyridoxal hydrochloride (1.0 mg/100 gm diet); (2) the restricted controls were given desoxypyridoxine (1.25 mg/100 gm diet) or were fed the complete purified diet ad libitum; (3) the controls were given desoxypyridoxine (1.25 mg/100 gm diet) or continued as before; (4) the diet controls were continued or given desoxypyridoxine (5.0 mg/day) by stomach tube in addition.

At the end of the 34th week all the animals remaining were sacrificed and histologic sections of heart, lung, liver, spleen, pancreas, adrenal, kidney, testes, and brain were examined for parenchymal damage or for lesions of the intrinsic vessels. Hematologic examinations of animals for all groups were performed at about the 25th week of the experimental period. During the 28th week the measurement of the sodium

[1] The vitamin mixture contained the following amounts in mg per 100 gm of diet:- thiamine hydrochloride 0.5, riboflavin 0.5, pyridoxal hydrochloride 1.0, calcium pantothenate 1.0, inositol 1.0, niacin 0.2, p-aminobenzoic acid 0.2, choline chloride 100.0, biotin 0.002 and 1–2 drops cod liver oil orally/week.

space, using radioactive sodium (Manery and Bale, '41) was made on selected rats in the different dietary groups. In the 32nd week xanthurenic acid excretion was studied by the method of Porter et al. ('47).

RESULTS

Growth. The body weights of the rats in all the groups reached a plateau about the 16th week of the experimental period (fig. 1); however, the mean body weights of the groups were quite different. The growth curve for the checker-fed diet controls was parallel to that exhibited by the controls, leveling at mean body weights of 420 and 360 gm, respectively. The mean body weight of the deficient group leveled at 220 gm. The food intake per rat per day at this time was 21 gm for the controls, 25 gm for the diet controls, 19 gm for the deficient, and 12 gm for the restricted controls.[2]

Typical signs of vitamin B_6 deficiency appeared in the deficient animals as early as one week after the administration of desoxypyridoxine. A fulminating diarrhea, scaly nose, paws, and tail, and, finally, edematous paws and convulsions were shown in this group. Because of the high mortality rate in these animals the analog was removed from the diet. The 9 rats that continued to receive the antivitamin were dead by the 10th week of the experimental diet. In all cases in which desoxypyridoxine was fed there was a loss in body weight with a recurrence of clinical manifestations. The addition of pyridoxal hydrochloride to the diet resulted in a sharp increase in growth, and if B_6-deficiency signs were present, an alleviation of the gross physical effects.

Blood pressure. Throughout the entire experiment, measurements of systolic blood pressure were made at approximately three-day intervals.[3] At least 6 determinations were

[2] The data from an earlier experiment (Olsen and Martindale, '52) are combined with this study. Casein, (Nutr. Biochem. Corp.) containing 70 μg pyridoxine hydrochloride per 100 gm, was used at a level of 18%, and the dextrose was added at the level of 68%.

[3] It should be noted that all recorded systolic blood pressure determinations were made on trained rats by a single trained technician who was continually and meticulously checked.

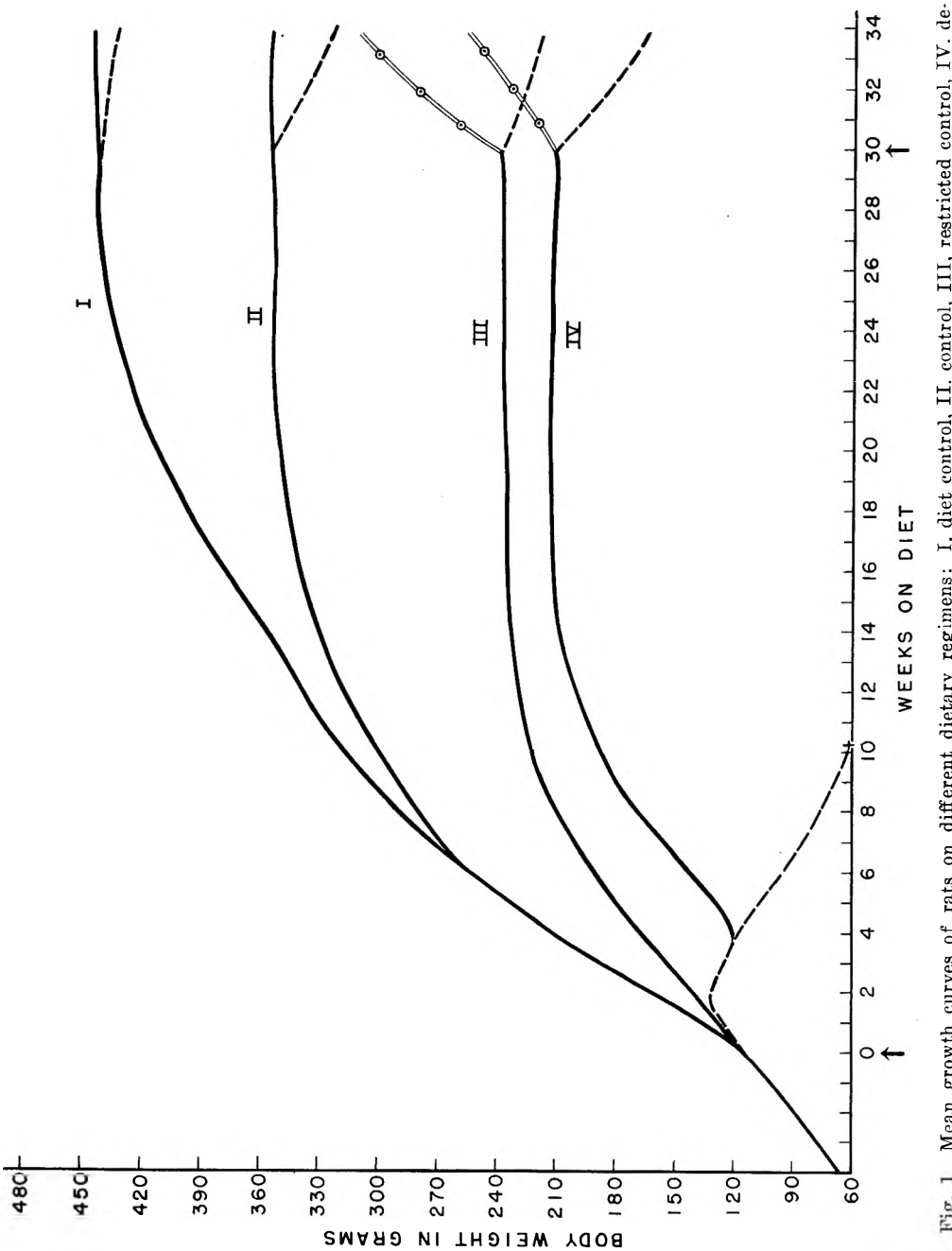

Fig. 1 Mean growth curves of rats on different dietary regimens: I, diet control, II, control, III, restricted control, IV, de-

made on each day on each animal. Using the average of these determinations a mean was calculated for each group. The systolic blood pressure for all rats throughout the conditioning period and at the start of the experimental period was 119 $\pm$ 1 (S.E.M.) mm of mercury. The three groups of controls — control, dietary control, restricted control — exhibited blood pressures within three standard deviations of this value during the 34 weeks of the investigation (table 1). The probability of any control value falling outside of this range was less than 0.001. The vitamin B_6 deficient group had a mean value of 134 $\pm$ 3 or a mean elevation of 15 $\pm$ 3 mm of mercury; this difference was highly significant ($P < 0.001$). These mean values were stabilized at about the 8th week of the experiment at which time the animals were considered young mature rats. A small group of deficient animals was continued on a supplement of desoxypyridoxine until death ensued. The systolic pressures in this group rose as high as 150 mm of mercury, with a mean of 148 $\pm$ 3 mm of mercury.

After 30 weeks on the experimental diets, the groups were subdivided into different dietary regimens, supplemented either with desoxypyridoxine or pyridoxal, which were continued for a period of 4 weeks. The mean changes from the previous blood pressure levels were calculated and, in essence, the effect of producing or repleting a deficiency in mature adults was determined (table 2). It will be noted that all of the control groups receiving the antimetabolite, desoxypyridoxine, exhibited a significant increase in systolic blood pressure. In this portion of the study the controls were not grouped together as the vitamin B_6 could not be removed from the laboratory checkers of the diet controls. No significant rise in blood pressure was elicited from the already deficient animals; however, the addition of pyridoxal to the diet of the deficient animals was followed within three to 4 days by a highly significant decrease (20 $\pm$ 3 mm of mercury) in systolic blood pressure.

Sodium space. The average sodium space of 7 B_6-deficient rats was found to be 26.4% $\pm$ 0.8. For the same number of

control animals, the mean sodium space was 23.8% ± 0.8. Since there was no significant difference in these two groups, further sodium space determinations in other dietary groups were not performed.

TABLE 1

Mean systolic blood pressure of control and deficient rats

GROUP	NO.	MEAN SYSTOLIC PRESSURE (mm Hg ± S.E.M.)
"Controls"	91	119 ± 1
— Diet Control	20	118 ± 1
— Control	51	119 ± 1
— Restricted Control	20	120 ± 1
Deficient	67	134 ± 3

TABLE 2

Changes in mean systolic blood pressure with different dietary regimens

GROUP	NO.	MEAN CHANGE IN SYSTOLIC PRESSURE (mm Hg ± S.E.M.)	P VALUE
Diet-control + desoxypyridoxine	10	+ 8 ± 2	< 0.01
Control + desoxypyridoxine	15	+ 14 ± 3	< 0.001
Restricted control + desoxypyridoxine	8	+ 10 ± 2	< 0.01
Deficient + desoxypyridoxine	18	+ 3 ± 3	> 0.2
Deficient + pyridoxal	16	— 20 ± 3	< 0.001

Xanthurenic acid excretion.—As further proof of the presence of the vitamin B_6 deficiency, xanthurenic acid excretion studies with and without tryptophan load, were performed. The data (table 3) are expressed in three ways: mg xanthurenic acid excreted per rat per day, mg xanthurenic acid excreted per 100 gm rat per day, and mg xanthurenic acid excreted per 100 gm rat per 100 mg DL-tryptophan fed per day. The results clearly show an increased excretion of xanthur-

TABLE 3

Urinary excretion of xanthurenic acid with and without tryptophane load [1]

	WITHOUT TRYPTOPHANE			WITH TRYPTOPHANE		
	NO.	mg x. a. per rat per day	mg x. a. per 100 gm rat per day	NO.	mg x. a. per rat per day	mg x. a. per 100 gm rat per day	mg x. a. per 100 gm rat per 100 mg DL-tryptophane
Controls	7	0.034 ± 0.011	0.016 ± 0.008	8	2.12 ± 0.346	0.628 ± 0.153	0.266 ± 0.063
Controls + desoxypyridoxine				3	14.20 ± 3.267	3.42 ± 1.685	0.743 ± 0.364
Restricted Controls	12	0.162 ± 0.039	0.093 ± 0.025	12	0.90 ± 0.220	0.521 ± 0.063	0.190 ± 0.040
Restricted Controls + desoxypyridoxine				5	24.6 ± 5.90	13.00 ± 0.76	2.77 ± 0.184
Restricted Controls + added pyridoxal				3	4.37 ± 0.890	0.857 ± 0.042	0.175 ± 0.014
Deficient	20	0.530 ± 0.071	0.251 ± 0.033	21	9.70 ± 1.28	4.40 ± 0.544	2.35 ± 0.323
Deficient + desoxypyridoxal				1	19.5	13.52	7.47
Deficient + added pyridoxal				4	4.30 ± 0.83	1.12 ± 0.073	0.134 ± 0.024

[1] The test load of tryptophane was 460 mg of the DL-form per rat. Values given are mg x. a. excreted per day ± S.E.M.. Underlined values differ from the controls at a level of $P < 0.001$. These tests were performed during the 32nd week of the experiment.

enic acid in the deficient animals either with or without added tryptophan.

Hematologic and histologic studies. Hematologic studies were done on a limited number of rats (48). The red cell counts and hemoglobin values for the controls, restricted controls, and deficient were similar and within normal limits. It must be noted that the white blood cells decreased significantly in the deficient animals ($8.3 \times 10^3/mm^3$) while the reticulocytes increased (2.6%) when compared to the controls ($15.4 \times 10^3/mm^3$ and 1.6% respectively); however, these same changes were found in the restricted control animals ($8.3 \times 10^3/mm^3$ and 2.6%). Under the dietary conditions of these experiments, anemia was not part of the deficiency syndrome.

Microscopic sections of the following viscera were examined for parenchymal damage and for lesions of the intrinsic vessels: heart, lung, liver, spleen, pancreas, adrenal, kidney, testis and brain. Aside from occasional parenchymal disease attributable to causes other than deficiency (such as infection) the organs were within normal limits in all groups and in no instance was sclerosis or other vascular lesions demonstrated.

DISCUSSION

The purified diet used in these experiments gave parallel growth effects as compared to the stock diet of laboratory checkers. This may be taken as evidence of the adequacy of the purified diet. The presence of a vitamin B_6 deficiency was shown by two criteria. In the first place, typical signs of the deficiency were produced both in rats fed a deficient diet and, also, by supplementing the diet with the antivitamin, desoxypyridoxine. These physical signs of the deficiency were reversed by the repletion of the diet with pyridoxal. Secondly, it has been established that the excretion of xanthurenic acid is elevated in vitamin B_6 deficiency, especially after a test dose of tryptophan. In the present experiment it can be seen that even without added tryptophan the deficient animals excreted more than 10 times the quantity of xanthurenic acid excreted by the controls. This difference was even more

marked when a tryptophan load was added. It should be noted that while these data are expressed on different bases, the more representative index would be obtained by taking into account the endogenous excretion of xanthurenic acid, body weight of the animal, and the amount of added tryptophan. Regardless of the manner of expression, the vitamin B_6-deficient animals excreted the larger amounts of xanthurenic acid. It is an interesting observation that in the toxemias of human pregnancy, where elevation of blood pressure and edema are primary findings, urinary xanthurenic acid levels are significantly elevated (Sprince et al., '51; Wachstein and Gudaitis, '52).

In all cases of vitamin B_6 deficiency, as evidenced by the criteria outlined above, there was exhibited a rise in the systolic blood pressure of the animal which could be reversed by the addition of pyridoxal hydrochloride to the diet. This could also be produced and reversed in older animals. In the case of the diet controls, desoxypyridoxine was fed by stomach tube, as pyridoxal could not be removed from the diet. The small elevation in blood pressure in this group probably was due to the fact that a deficiency of sufficient degree was not produced because of the large amount of vitamin B_6 in the checkers.

The histologic survey failed to reveal any evidence of arterial or arteriolar disease and no significant irreversible disease process. Calder ('44), however, has reported lesions in the kidneys of rats on a diet deficient in the vitamin B_2 complex. Rinehart and Greenberg ('49) demonstrated the occurrence of arteriosclerotic lesions in the monkey made deficient of pyridoxine. These lesions were not developed in monkeys subjected to other dietary deficiencies. Agnew ('49) reported the presence of hematuria and renal lesions in the hooded rat, but not in the albino rat. In the present experiment, using albino rats, there was no evidence of hematuria. The absence of lesions of any type might be expected as the deficiency could be reversed by restoration of the vitamer to the diet.

The occurrence of an anemia, usually of the microcytic type, has been reported in a number of species in vitamin B_6 deficiency (Hawkins et al., '52). Agnew ('49) reported a microcytosis with increased number of red cells in both the albino and hooded rat deficient in vitamin B_6. Increased red cell counts were found by Hawkins et al. ('52) in rats which had been deprived of vitamin B_6. The red cell counts and hemoglobin values of the deficient animals in the present experiment did not differ significantly from the controls or from the restricted controls. Hematologic values of the deficient animals were essentially the same as those exhibited by the restricted controls.

SUMMARY

A vitamin B_6 deficiency was produced in male albino rats by the use of the antivitamin, desoxypyridoxine, and a deficient diet. This was evidenced by the typical clinical signs of deficiency and an increased urinary excretion of xanthurenic acid.

A significant increase in systolic blood pressure occurred in animals that were made deficient in vitamin B_6 by either simple dietary depletion or the use of desoxypyridoxine. This elevation in blood pressure could be rapidly reduced to normal limits and the clinical signs of the deficiency reversed by the dietary use of pyridoxal hydrochloride. Old as well as young animals showed this pattern.

Hematologic and histologic studies failed to reveal any significant changes, and in no case were vascular lesions demonstrated.

The extracellular fluid volume of the deficient animals was well within the normal limits.

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STUDIES ON CHRONIC VITAMIN B_6 DEFICIENCY IN THE RAT

II. CHANGES IN TISSUE METABOLISM

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ONE FIGURE

In a previous communication we reported the changes in the intact rat with chronic vitamin B_6 deficiency (Olsen and Martindale, '53). In addition to these over-all changes, it seemed of interest to describe further tissue effects. The vitamin B_6 content of tissues decreased in deficient rats (Snell et al., '42) and the concentration of certain enzymes also was found to be lowered in various organs of vitamin B_6 deficient rats (Meister et al., '53, and Roberts et al., '51). Beare et al. ('53) have found a loss of fat in the carcass of vitamin B_6 deficient animals. The heart and kidney were shown by Agnew ('51) to be hypertrophied in both the deficient hooded and albino rat. All of these studies were performed on a deficiency of relatively short duration. In the present report the organ weights per 100 gm of rat and the vitamin B_6 content of liver and kidney were studied. In addition, the oxygen uptake of tissue preparations with and without addition of various substrates was also investigated.

EXPERIMENTAL

Weanling, male albino rats of the Wistar strain were placed on various control and vitamin B_6-deficient diets, with and

without the antivitamin, desoxypyridoxine. Details of the dietary regimens and experimental conditions have been previously described (Olsen and Martindale, '54). The 4 major diet groups were: (1) control — complete purified diet; (2) diet control — Purina laboratory checkers; (3) restricted control — complete purified diet with intake restricted to serve as weight controls for deficient animals; (4) deficient — complete, purified diet with the pyridoxal replaced by the antimetabolite, desoxypyridoxine, for 4 weeks; after this period the desoxypyridoxine was removed and the animals maintained on a deficient diet. At the end of 38 weeks the animals were killed by decapitation and exsanguinated. The following organs were removed, cleaned of adipose and connective tissue, blotted on filter paper, and weighed immediately on an analytical balance: adrenal, heart[1], liver, and kidney. A portion of liver and kidney was taken for microbiological assay of "vitamin B_6" by the method of Atkin et al. ('43) using *Saccharomyces carlsbergensis*. The remaining portions of these two organs were used in oxygen uptake experiments. Broken cell preparations of liver and kidney were prepared by blending the tissues with one to 4 volumes of 0.05 *M* phosphate buffer, pH 7.84, per gm of wet tissue and then squeezing through muslin. The rate of oxygen uptake of 1.0 ml aliquots of the tissue preparations to which had been added 1.0 ml of buffer containing substrate, additives, or both, was measured at 37.5°C. in the usual Warburg apparatus. The difference between the oxygen uptake in the presence of substrate and that of a suitable control was considered a measure of the uptake of the substrate.

RESULTS

The data for the relative organ weights are expressed as gm or mg wet weight of organ per 100 gm rat (table 1). In the three control groups, controls, diet controls, and restricted controls, there was no statistically significant difference in

[1] The major vessels and auricles were removed and the ventricular mass was determined.

TABLE 1

Organ weights of control and B_6 deficient rats

Mean organ weights expressed on basis of 100 gm rat — means are followed by S.E.M. The values shown in italic figures are significantly different from the control group at the $P < 0.001$ level.

GROUP	NO.	ADRENALS	HEART	LIVER	KIDNEYS
		mg	*gm*	*gm*	*gm*
Diet control	10	9.3 ± 0.6	0.221 ± 0.003	3.2 ± 0.1	0.64 ± 0.03
Diet control + desoxypyridoxine	10	9.6 ± 0.8	0.229 ± 0.003	3.1 ± 0.1	0.64 ± 0.40
Control	14	9.7 ± 0.5	0.215 ± 0.005	2.9 ± 0.1	0.63 ± 0.02
Control + desoxypyridoxine	8	*12.6* ± 0.6	0.225 ± 0.010	3.3 ± 0.1	0.71 ± 0.04
Restricted control	5	11.3 ± 0.6	0.260 ± 0.010	3.3 ± 0.1	0.78 ± 0.05
Restricted control + desoxypyridoxine	7	*17.4* ± 0.7	*0.261* ± 0.015	3.3 ± 0.1	0.80 ± 0.05
Restricted control + pyridoxal	5	10.4 ± 0.6	0.258 ± 0.016	3.1 ± 0.1	0.78 ± 0.06
Deficient	13	*15.5* ± 0.8	*0.281* ± 0.009	*3.5* ± 0.1	*0.91* ± 0.08
Deficient + desoxypyridoxine	9	*14.9* ± 0.8	*0.277* ± 0.011	3.4 ± 0.2	*0.88* ± 0.12
Deficient + pyridoxal	14	*13.8* ± 1.1	*0.279* ± 0.017	3.2 ± 0.2	0.80 ± 0.05

any of these parameters. However, in the deficient animals all of the organs studied, adrenal, heart, liver and kidney, showed significant increases in relative wet weight ($P < 0.001$) as compared to the control groups.

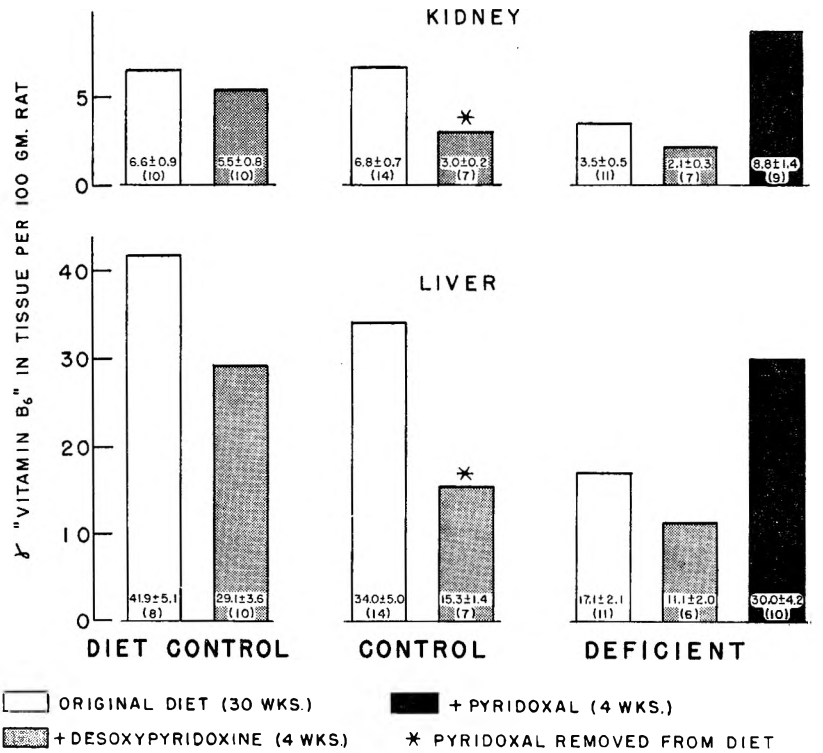

Fig. 1 "Vitamin B_6" content of liver and kidney of the experimental animals using *Saccharomyces carlsbergensis* as the assay organism. Number of samples in each group in parentheses. Mean values followed by S.E.M.

Microbiological assays were performed on representative portions of hepatic and renal tissues from rats on the various dietary regimens. The data are expressed as total micrograms of "vitamin B_6" for the whole organ per 100 gm rat (fig. 1). While there is no difference between the means of the two control groups, the "vitamin B_6" content of both liver and kidney of the deficient rats was significantly lowered

($P < 0.001$). After being placed on a complete diet with added pyridoxal for 4 weeks, the mean values for the liver and kidney of deficient rats returned to within normal limits. In addition, the control group, when placed on a deficient diet with desoxypyridoxine, exhibited a striking decrease in the "vitamin B_6" content of liver and kidney.

The oxygen uptakes of broken-cell preparations of liver and kidney are expressed as micromoles of oxygen utilized in 120 minutes per gm wet weight of tissue (table 2). The data show a marked decrease in basal oxygen consumption in the tissue from deficient animals; in the case of liver a mean decrease of 30.4% $\pm$ 6.7 from the controls ($P < 0.001$) and in kidney a decrease of 23.1% $\pm$ 2.4 from the controls ($P < 0.01$). When pyridoxal was added to the preparations from deficient animals, the oxygen uptake was increased to approximately the control levels. Amino acids and the amines derived from them were also added as substrates; the compounds studied were DL-phenylalanine, DL-leucine, L-tyrosine, L-tryptophan, β-phenylethylamine, isoamylamine, tyramine and tryptamine. The final concentration of substrate in all cases was 0.02 *M*. The rates of oxidation of the added amino acid or amine were similar for both the controls and deficient animals. The only difference was a slight tendency to a lowered oxidation of amino acids in kidney preparations of deficient animals. Experiments were performed using as substrates (1) amino acids or amines with pyridoxal, and (2) chemical complexes of pyridoxal and amino acids (or amines) synthesized in the laboratory (Heyl et al., '48, '48a). The oxygen uptakes were usually depressed when the synthetic complexes were added to the tissue preparations of all animals. To clarify the results, the data are expressed as the differences in oxygen uptake between the substrates "pyridoxal + amino acid or amine" and "synthetic pyridoxal amino acid (or amine) complex" (table 3). It is noted that in every experiment the difference in oxygen uptake was smaller in the preparations of tissues from the deficient animals. In no case was the rate of oxidation of the pyridoxylamino acid (or amine)

TABLE 2

Oxygen uptake of tissue preparations alone and with added pyridoxal

The values are expressed as micromoles of oxygen uptake/120 minutes/gm wet weight of tissue ± S.E.M.

GROUP	NO.	LIVER		KIDNEY	
		Alone	+ Pyridoxal	Alone	+ Pyridoxal
Diet control	23	35.3 ± 0.9	34.2 ± 1.1	28.2 ± 0.9	28.9 ± 1.0
Diet control + desoxypyridoxine	10	37.5 ± 1.8	36.5 ± 2.2	29.3 ± 1.4	28.7 ± 0.7
Control	14	35.7 ± 1.1	35.4 ± 1.0	28.3 ± 1.0	28.4 ± 0.8
Control + desoxypyridoxine	8	29.2 ± 2.1	32.1 ± 0.9	24.3 ± 1.7	26.1 ± 1.3
Deficient	12	25.6 ± 2.0	29.3 ± 1.8	22.9 ± 1.2	26.3 ± 1.2
Deficient + desoxypyridoxine	8	24.3 ± 2.2	29.7 ± 1.5	22.9 ± 1.5	26.6 ± 1.1
Deficient + pyridoxal	11	36.0 ± 1.7	36.3 ± 1.1	26.7 ± 0.9	26.4 ± 1.0

TABLE 3

Difference in oxygen uptake of pyridoxal + amino acid (or amine) and pyridoxylamino acid or pyridoxylamine

SUBSTRATE	LIVER			KIDNEY		
	No.	Control	Deficient	No.	Control	Deficient
DL-phenylalanine	6	19.2 ± 1.1	16.2 ± 0.5	5	36.5 ± 1.4	18.0 ± 1.0
L-tyrosine	5	8.1 ± 0.2	6.1 ± 1.0	4	7.6 ± 0.4	3.8 ± 0.5
β-phenethylamine	4	10.8 ± 0.4	9.9 ± 0.9	4	12.4 ± 0.5	8.2 ± 0.9
Tyramine	6	16.2 ± 0.7	15.8 ± 0.5	5	5.7 ± 0.5	3.7 ± 0.6

complex higher than that of the mixture of the two components.

DISCUSSION

In vitamin B_6 deficiency the present data indicate that there is a significant increase in the wet weight of adrenal, heart, liver and kidney. This confirms the observation by Agnew ('51) of hypertrophy of the heart and kidney in vitamin B_6-deficient rats. The fact that there is no difference in the controls and restricted controls (restricted in food intake to serve as weight or inanition controls for the deficient animals) indicates that the hypertrophy was not due to starvation. When the deficient animals were given pyridoxal in the diet for a period of 4 weeks, the organ weights on the basis of a 100 gm rat were lowered. This apparent decrease in size might have been caused by a diminution in organ weight ratios but actually was due to the rather rapid over-all growth of the animals on the complete diet. In the restricted controls, the addition of pyridoxal to the diet showed no significant changes in the organ weights studied. However, when the control and restricted control animals were given desoxypyridoxine for a period of 4 weeks, the adrenal and heart showed hypertrophy and there was a tendency toward the same in liver and kidney. These changes could be only partially explained by a loss in total body weight during the period.

The microbiological method used in the investigation gives a value of "vitamin B_6" content of tissues. Since the B_6 group consists of at least 4 vitamers — pyridoxine, pyridoxal, pyridoxamine, and pyridoxal phosphate — no definite statement can be made concerning any individual component. The data indicate that the deficient animals showed a lowered content of vitamin B_6 in the liver and kidney as compared to control animals ($P < 0.001$). This is in accord with the earlier work of Snell et al. ('42). When pyridoxine was added to the diet of the deficient group for a period of 4 weeks there was a return to normal levels and, in the case of the kidney, an actual increase or overstorage in the tissue. A similar rebound phe-

nomenon has been noted in studies on repletion after vitamin A deficiency (Eden and Moore, '51). The observed reduction of approximately 50% in the content of vitamin B_6 in hepatic and renal tissues of deficient animals would be expected to produce a decrease in its activity in decarboxylation and transamination reactions.

Broken cell preparations of liver or kidney from diet control or control animals showed no significant differences in their oxygen uptake in the presence or absence of added pyridoxal. Similar preparations from deficient animals showed a mean decrease of over 30% for liver and over 20% for kidney. However, when pyridoxal was added to the broken-cell tissue preparation the oxygen uptake was increased almost to the control level. A slight decrease was noted in the rate of oxidation of amino acids in preparations from renal tissues of deficient animals. This substantiates in part the finding of Armstrong et al. ('50) that homogenized kidney preparations from vitamin B_6-deficient rats had only one-third as much D-amino acid oxidase activity as preparations from normal animals. If, however, the food intake of control animals was restricted to that of the vitamin B_6-deficient animals, there was no significant difference in oxidation. Although no pair-fed controls were studied in the present investigation, it should be noted that the restricted controls had approximately the same mean kidney weight and mean B_6 content as the controls and would thus be expected to have the same approximate oxygen uptake.

Since it is postulated in the transamination and decarboxylation reactions that the amino acid forms an intermediate complex with vitamin B_6, it was of interest to investigate such complexes in oxygen uptake studies. Complexes of pyridoxal with phenylalanine and tyrosine, and their respective derived amines were studied. The results of these experiments were compared to studies using a mixture of pyridoxal and the corresponding substrate. In every case, the oxygen uptake of mixtures was higher than the synthetic complex. This might lead one to the conclusion that either the complex

plays no important role in bodily reactions or that its metabolic half-life was extremely small. In the case of the deficient tissue preparations this difference was markedly reduced as compared to the normals. The decrease was for the most part due to the lowered level of "free vitamin B_6" in the tissues of the deficient animals.

In correlating the changes in the intact animal with chronic vitamin B_6 deficiency (Olsen and Martindale, '54) with the present tissue changes, the most striking observation is that of an elevation in systolic blood pressure. Since no renal or adrenal vascular lesions are found and the extracellular fluid volume is within normal limits, this hypertension seems to be unique.

It must be noted that the elevation of systolic blood pressure was only moderate as compared to that produced by the other mechanisms. This reversible, mild hypertension is not the result of structural change but seems due to some easily reversible metabolic phenomenon associated with B_6 deficiency. Instead of being a primary effect, this elevation of systolic blood pressure may only be of indirect origin and of secondary importance. It is difficult and dangerous to separate one parameter from a syndrome complex and postulate a mechanism. For example, the hypertension associated with acute choline deficiency (Best and Hartroft, '49; Handler and Bernheim, '50) or pteroylglutamic acid (Lee et al., '52) is basically the result of renal damage, while that in pantothenic acid deficiency is a consequence of adrenal pathology (Lewis and Page, '53; Deane and McKibbin, '46; Terroine and Adrian, '50).

On the other hand, vitamin B_6 has been shown to be of importance in transamination and decarboxylation reactions in the body. It has further been shown that amines or amine-containing compounds play an important role in hypertension. If one assumes that amines react with vitamin B_6 in a similar manner to amino acids, the reaction might be used by the body to detoxify amines. For example, phenethylamine could form a Schiff base type of complex with pyridoxal. This tautomeric complex could be broken to give either the original

two components or pyridoxamine and phenacetaldehyde. The result of the latter cleavage would be the production of a non-pressor aldehyde from a pressor amine; the vitamin B_6 component going back into the cycle. In vitamin B_6 deficiency there would be a lack of the hypothetical complexing agent and the amines or amine-containing compounds (held by some to be formed in the kidney) would accumulate and exert a continued hypertensive action. It has previously been demonstrated that synthetic complexes of pyridoxal with pressor amines have no hypertensive effect *in vivo*. In addition, these complexes are utilized by broken cell preparations of renal and hepatic tissue.

SUMMARY

Tissue changes in the chronic vitamin B_6-deficient rat were studied. There was an increase in the wet weight of the adrenal, ventricular mass, liver and kidney of the deficient animal when compared to the normal or inanition control. When the antivitamin, desoxypyridoxine, was added to the diet of all rats, even if for a short time, the same tendency to hypertrophy was noted.

Microbiological assay for "vitamin B_6" content was performed on portions of liver and kidney. The B_6 content of these tissues was lower in the deficient than in the control animals.

Oxygen consumption studies by the Warburg technique revealed a 30% decrease by broken-cell preparations of hepatic tissue from the deficient animals and a 20% decrease by renal tissue preparations. The addition of pyridoxal restored these values to approximately normal. The addition of certain amino acids and amines as substrates resulted in similar rates of oxidation for preparations from both the deficient and the control animals. The addition of pyridoxylamino acid or pyridoxylamine complexes gave lower rates of oxidation when added to the tissue preparations than did mixtures of pyridoxal and the amino acid or amine. The possible metabolic role of the complexes are discussed.

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EFFECT OF VARYING THE INTAKE OF CALCIUM PANTOTHENATE OF RATS DURING PREGNANCY

I. CHEMICAL FINDINGS IN THE YOUNG AT BIRTH [1,2]

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The recent review by Lowry ('52) concerned with the biochemical evidence of nutritional status for ascorbic acid, thiamine, and riboflavin points out how meager our information still is with respect to the chemical and structural changes which occur during the onset of nutritional deficiences. This paper also illustrates how different the vitamin intake of the animal must be to accomplish tissue saturation on the one hand and, on the other, to be a handicap to the organism due to chemical or structural changes, resulting from a vitamin shortage.

Earlier studies from this laboratory concerned with the pantothenic acid needs of the rat during reproduction have revealed that there are wide differences in the amount of this vitamin present in the tissues of the rat depending upon the composition of the maternal diet. It was therefore decided

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to investigate the influence of varying the pantothenic acid intake of the rat during pregnancy upon the concentration of additional blood and tissue components of the young at birth. Information has also been sought as to the concentration of pantothenic acid which may be present in the blood and total tissues of the young at a time when chemical or structural changes are detectable.

Total pantothenic acid activity of the tissues of the young and the dams has been measured directly following parturition. Blood pantothenic acid, serum ascorbic acid, serum alkaline phosphatase and blood pyruvic acid have been determined for young rats at birth. Simultaneously, histological examinations of liver, adrenal, intestine and the tibia of the young were carried out. The present paper reports the results of the chemical studies completed to date. Microscopic data will be reported separately (Chung, Northrop, Getty and Everson, '54).

EXPERIMENTAL

Albino rats which were 28 days old were selected from an inbred colony of the Wistar strain. These animals received the customary stock diet consisting of mixed grains and dried whole milk, plus supplements of fresh beef and carrots. Females producing first litters of normal size and of satisfactory appearance and birth weight were distributed into 4 groups: Group I was continued on the stock ration; Group II received a synthetic ration plus a vitamin mix containing all of the known vitamins except pantothenic acid and ascorbic acid; Group III received a ration similar to that of Group II except that the vitamin mix provided 100 μg of calcium pantothenate per day; Group IV received the same ration plus 1 mg of calcium pantothenate daily. The composition of the synthetic diet and of the vitamin mix are given in table 1. First litter young were sacrificed within a few hours after birth. The females were remated for second pregnancies one week following the first parturition. Immediately following the delivery of the second litters and before the young had an op-

portunity to nurse, the newborn were removed from the female's cage. The weight and sex of the rat was recorded.

Total pantothenic acid activity of the blood of young produced by females in each of the 4 groups was determined by microbiological assay using *Lactobacillus arabinosus* as the test organism. The same technique was used in determining

TABLE 1

Composition of the synthetic ration

Vitamin-free casein	25%
Dextrose	55%
Swiftening	15%
Salts [1]	5%
Vitamin mix fed daily per rat	
Choline chloride	10 mg
Inositol	5 mg
p-aminobenzoic acid	100 μg
2-methyl-Naphthoquinone	250 μg
Thiamine hydrochloride	150 μg
Riboflavin	100 μg
Niacin	500 μg
Biotin	2.5 μg
Folic acid	6 μg
Pyridoxine hydrochloride	50 μg
B_{12}	0.5 μg
Fat soluble vitamins	
Cod liver oil: 9 U.S.P. units vitamin D 90 U.S.P. units vitamin A	
Alpha tocopherol — 750 μg	

[1] Richardson and Hogan — J. Nutrition, *32:* 459, 1946.

the total pantothenic acid content of fetal tissues, maternal liver and carcass. In all cases these tissues were incubated with chicken liver enzyme and intestinal phosphatase at pH 8.5.

Total ascorbic acid, determined for serum, was estimated by the Lowry, Lopez and Bessey microadaptation of the dinitrophenylhydrazine method of Roe and Kuether (Lowry, Lopez and Bessey, '45; Roe and Kuether, '43). The alkaline phosphatase activity of the serum of the young was deter-

mined by the Bessey, Lowry and Brock procedure ('46). Pyruvic acid concentrations of the blood were determined by the micromethod of Tsao and Brown ('50).

RESULTS AND DISCUSSION

Transferring young females immediately following the the birth of their first litters from the customary stock ration to synthetic rations containing zero, 100 μg or 1 mg quantities of calcium pantothenate had a marked effect upon the reproductive performance of the females. Animals which received no pantothenic acid from approximately one week prior to pregnancy and throughout the entire gestation period produced inferior litters in all instances. The average number of young born to such females was 4.2 with an average birth weight of 3.7 gm (table 2). In several cases no young were carried to term and in many instances parturition was delayed.

The addition of 100 μg of calcium pantothenate per day to the same ration increased the size of the young and the number of animals per litter. Such females delivered their young on time although an occasional litter was poor. The further addition of calcium pantothenate to furnish 1 mg daily, improved the birth weight of the young slightly but did not alter the litter size. While the majority of young produced by group IV were considered excellent, an occasional animal resorbed her entire litter. The performance of the females on the synthetic ration was not considered equal to that of the stock females although many excellent litters were produced by females receiving the higher intake of calcium pantothenate.

Sufficient blood was obtained from a number of young produced by each of the 4 groups of females to allow for 4 individual checks of the total pantothenic acid activity of this tissue. Results from these assays revealed that the blood of young born to females receiving the stock diet averaged 450 μg of pantothenic acid per 100 ml. Values within the group

TABLE 2

Chemical findings in rats resulting from differences in pantothenic acid intake of the maternal rat during pregnancy [1]

RATION	LITTER SIZE [2]	BIRTH WEIGHT [2]	PANTOTHENIC ACID CONTENT OF TISSUES			PANTOTHENIC ACID CONTENT OF BLOOD OF YOUNG	ALKALINE PHOSPHATASE OF SERUM OF YOUNG	ASCORBIC ACID OF SERUM OF YOUNG	PYRUVIC ACID OF BLOOD OF YOUNG
			Maternal liver	Maternal carcass	Newborn carcass				
		gm	*μg/gm*	*μg/gm*	*μg/gm*	*μg/100 ml*	*nitrophenol units*	*mg/100 ml*	*mg/100 ml*
Stock diet	11.2	5.2	67.8	7.5	36.9	450	14.6	5.85	1.04
450–600 μg calcium			(64.6–70.9)	(7.0–7.9)	(29.1–45.8)	(342–570)	(9.9–19.5)	(4.13–8.38)	(0.29–1.88)
pantothenate daily	(28)		(10)	(10)	(40)	(50)	(58)	(67)	(18)
Synthetic diet	4.2	3.7	40.7	4.4	6.7	295	12.0	4.12	2.79
No calcium panto-			(38.6–43.6)	(3.2–5.0)	(5.1–7.6)	(108–447)	(7.0–16.9)	(2.47–6.00)	(0.78–6.33)
thenate	(35)		(10)	(10)	(25)	(20)	(20)	(20)	(23)
Synthetic diet	7.8	4.6	54.6	5.5	15.8	502	11.8	4.58	....
100 μg calcium			(45.0–62.4)	(4.8–6.0)	(12.6–24.8)	(320–678)	(7.7–15.8)	(3.19–7.31)	....
pantothenate daily	(17)		(10)	(10)	(25)	(27)	(40)	(42)	....
Synthetic diet	8.0	5.1	72.1	9.2	72.1	2204	12.1	4.22	1.63
1 mg calcium			(64.8–80.5)	(8.5–10.4)	(60.1–75.7)	(1709–2575)	(9.2–15.8)	(3.10–6.08)	(0.42–2.25)
pantothenate daily	(18)		(10)	(10)	(24)	(15)	(42)	(44)	(20)

[1] Average values listed first; range given below; third value indicates number of animals examined in each group.

[2] Number of litters included in litter size and birth weight values.

varied considerably. The intra-group variation was not found to be due to sex or size differences.

The majority of young born to females deprived of dietary pantothenic acid throughout pregnancy possessed less vitamin per unit of blood than was true for young produced by stock females. In some instances the concentration was as little as 100 μg per 100 ml, while other animals of the group exhibited concentrations equal to those of the control group. Poor condition of the young did not always correlate with a low blood value.

The presence of 100 μg of calcium pantothenate in the vitamin mix allowed for the production of young with blood pantothenic acid values equal to those of the stock animals. When the female's diet was supplemented with as much as 1 mg of calcium pantothenate daily, the concentration was greatly increased. In certain animals this amount was 8 times that of stock animals believed to be well nourished in every respect. This finding suggested that some handicap was being encountered in removing this substance from the blood.

It will be observed from table 2 that the amount of pantothenic acid present in the maternal liver, the carcass and the entire fetus at birth was directly related to the quantity of pantothenic acid present in the maternal diet during pregnancy. The average concentration of the vitamin per gram of fresh fetal tissue for young of females devoid of dietary pantothenic acid was 6.7 μg, or approximately 23 μg per individual animal. If the maternal intake of calcium pantothenate was 100 μg daily throughout the gestation period the young at birth possessed approximately 16 μg of pantothenic acid per gram of fresh tissue. As the maternal diet provided larger amounts of the vitamin, additional quantities were transferred to the young until saturation of the tissues occurred when the maternal diet included 1 mg of the vitamin daily (Bjorck, Lewis and Everson, '45).

While the quantity of pantothenic acid present in the hepatic tissue of the mothers varied with the dietary intake of this factor, the amount of change encountered was consider-

ably smaller than that for fetal tissue. Those females which were unable to produce litters of normal size and birth weight possessed livers averaging 40 μg of pantothenic acid per gram of tissue, an amount which was approximately 56% of the saturation value.

Since earlier work by Nelson and her associates ('50) indicated that numerous changes in the endochondral ossification of the tibia occurred in rats suffering from acute pantothenic acid deficiency, it was thought possible that differences in alkaline phosphatase activity of the serum of the young might exist within the groups of animals being studied. It will be apparent from table 2 that no such differences were encountered. Young born to stock females which appeared in good condition at birth possessed serum concentrations equal to approximately 15 nitrophenol units [4]. Variation within the 58 young examined in this group was large and exceeded that found for the 4 groups under study. It would, therefore, appear that serum alkaline phosphatase activity is of no value in evaluating nutritional status from the standpoint of pantothenic acid stores.

The concentration of ascorbic acid in the serum of the newborn rat was likewise investigated, since it was thought that metabolic changes brought about by the withdrawal of pantothenic acid from the maternal diet might result in variations in the blood ascorbic acid of the young. While the data summarized in table 2 disclosed no effect upon serum ascorbic acid of the young from varying the pantothenic acid intake of the females, the concentrations of this vitamin were interesting. An average concentration of approximately 6 mg% was observed for young of stock females. Considerable variation in blood ascorbic acid was encountered among animals of the same dietary origin. While a small number of low ascorbic acid values were encountered among the animals ex-

[4] One unit is the amount of phosphatase activity per liter of serum required to liberate one millimole of nitrophenol per hour from sodium paranitrophenyl phosphate.

amined, such low concentrations were more common among animals receiving the synthetic diet than the stock ration.

Figures obtained in the present study are of interest when compared with data reported for adult rats by Todhunter and McMillan ('46). The latter workers observed that the ascorbic acid content of the plasma of adult males was 0.87 mg per 100 ml while that of females was only 0.33. In the current study no such difference was encountered between male and female rats at birth. Serum ascorbic acid values apparently are of no assistance in recognizing differences in the nutritional state of animals from the standpoint of pantothenic acid stores.

Blood pyruvic acid values taken at birth have been completed for a small number of young which were produced by females receiving the stock diet and synthetic rations. The results of these analyses suggest that the withdrawal of pantothenic acid from the diet of the female approximately one week before the initiation of pregnancy resulted in some elevation of blood pyruvate in the young at birth. High pyruvic acid values were not observed, however, for all animals in this group. Young born to females receiving the stock diet which provided between 450 and 600 μg of pantothenic acid daily, 150 μg of thiamine, and generous intakes of all other members of the B complex, averaged 1.04 mg of pyruvic acid per 100 ml of blood. There was wide individual variation encountered among the 18 animals tested. Examination of the blood of young produced by females deprived of pantothenic acid revealed an average pyruvic acid concentration of 2.79 mg %, and in 18 of 23 cases the amount of pyruvic acid present exceeded the highest value encountered among the stock animals. While the young produced by females receiving the synthetic diet plus 1 mg of calcium pantothenate daily exhibited lower concentrations of pyruvic acid than when the vitamin was omitted from the female's diet, a few animals included in this group apparently had more pyruvic acid in the blood than any animals fed the stock ration. The fact that the general performance of the females receiving the syn-

thetic diet supplemented with 1 mg of calcium pantothenate per day was not equal to that of the stock ration makes these differences in pyruvic acid especially interesting.

SUMMARY AND CONCLUSIONS

The effect of altering the pantothenic acid intake of the maternal rat during reproduction upon the concentration of several blood constituents of the young at birth and upon the pantothenic acid content of the maternal and fetal tissues at parturition has been studied. The females received a stock diet or synthetic rations furnishing zero, 100 μg or 1 mg amounts of calcium pantothenate daily.

It was observed that if the maternal diet supplied no pantothenic acid from the initiation of pregnancy, small litters of undersized young were produced. The tissues of such young at birth contained approximately 9% as much pantothenic acid as was present in young of mothers receiving 1 mg of the vitamin. Blood concentrations for pantothenic acid were reduced in this group of young and some accumulation of pyruvic acid was encountered.

The presence of 100 μg of calcium pantothenate in the maternal diet supported blood pantothenic acid values of the young equal to those of animals whose dams received the stock ration. Similarly, 100 μg of calcium pantothenate in the maternal diet resulted in normal pyruvic acid values of the young although the amount of pantothenic acid deposited in the fetal tissues at birth was still considerably less than that of rats produced by stock females.

Serum values for ascorbic acid and alkaline phosphatase were not altered by the differences in composition of the maternal diets.

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IRON FROM GASTROINTESTINAL SOURCES EXCRETED IN THE FECES OF HUMAN SUBJECTS

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ONE FIGURE

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The amount of iron absorbed from a single food has been measured in two ways: by feeding radioactive iron incorporated in a test food (Moore and Dubach, '51) and by feeding a basal diet, first without and then with a test food (McMillan and Johnston, '51). Neither method can be used to measure the amount of iron absorbed from an entire diet. For this, information about the amount of gastrointestinal iron in the feces is necessary. The amount of iron absorbed would then be calculated by subtracting from the iron in the food the residual food-iron in the feces. The latter value would be found by subtracting from the total iron in the feces the amount of iron from gastrointestinal sources.

An experiment on human beings concerned with finding the amount of iron from gastrointestinal sources was conducted by Dubach et al. ('49). Following intravenous injections of radioactive iron into 4 human subjects, the daily fecal excretion for 140 days after the isotope reached a constant blood level amounted to an average of 0.01% of the injected dose. In order to interpret this in terms of the total amount of gastrointestinal iron excreted in the feces, the proportion of the total circulating hemoglobin iron that was radioactive was calculated. The authors state that: "Assuming complete

mixing of isotopic iron with total hemoglobin iron, the total fecal excretion of iron derived from hemoglobin was calculated to be 0.2 to 0.9 mg/day for the normal subjects."

The present authors used two approaches for determining the amount of gastrointestinal iron in the feces of human subjects: (1) by the extension to zero of a regression line of fecal-iron on food-iron at three intakes as near to zero as possible, (2) by finding that percentage of absorption which, when applied to three levels of intake, resulted in the most nearly constant value for gastrointestinal iron. The latter approach was based on two suppositions: the one that the gastrointestinal iron in the feces remains constant regardless of the iron intake, the other that the percentage of iron absorbed remains constant (as nearly as can be measured) over a narrow range of iron intake.

PROCEDURE

Eight women 23 to 37 years of age served as subjects; they varied in height from 145 to 168 cm and in weight from 48.6 to 64.4 kg. The hemoglobin values for the subjects were 12 gm or more per 100 ml of blood except for two subjects (C.B. and B.O.), who participated only early in the study and for whom determinations were not made. Three experimental 8-day periods were each preceded by three days during which the subjects consumed a diet of self-selected foods but avoided foods high in iron. Preliminary experimental work showed that after such a three-day period of lowered iron intake two days rather than 4 days of adjustment on the unpalatable experimental diet were sufficient. Thus 6 days remained in each main experimental period. Each subject participated in the study following the end of menstrual periods, thus possible variations in absorption which might occur during the menstrual cycle were avoided. All 8 individuals did not continue as subjects throughout the study; 5 completed the diet for all three experimental periods, two completed the first period only and one completed the second and the third periods.

The diets used during the three experimental periods were planned to contain 1, 2 and 3 mg of iron per day, respectively. The low intakes were necessary to warrant the making of calculations concerning the amount of iron excreted in the feces at an intake of zero. The diet containing 1 mg of iron consisted of 2 kg of milk daily and sugar, dissolved in glass-distilled water, in amounts to meet the remainder of the caloric needs of the individual. The subject with the highest energy needs was given heavy cream in place of part of the sugar. During the second period the iron intake was increased by replacing part of the sugar with bread, applesauce and peaches. In the third period egg and additional peaches were included to further increase the intake of iron. Twenty-five milligrams of ascorbic acid was given daily during the first period, but was omitted during the other periods because ascorbic acid was added to the peaches and applesauce before freezing. The subjects drank some of the milk between meals and in the evening because they found that the drinking of 2 kg of milk during the three meal periods was difficult.

Particular care was taken to eliminate sources of iron contamination. Glass, aluminum or pottery dishes and silver or stainless steel knives and spoons were used for preparing and serving the food. Glass-distilled water was used for cooking and drinking purposes. The subjects were asked to avoid licking stamps, placing foreign objects in their mouths and eating without removing lipstick. Tooth powder made in the laboratory of sodium chloride and sodium bicarbonate was furnished.

A conscious effort was made to keep iron content of the diet constant. The milk, purchased from one dairy, was analyzed at intervals throughout the experimental period and was found to remain unchanged in iron content. Several brands of sugar were analyzed and that with the lowest iron content was used. Three to 6 loaves of bread were made at one time from unenriched flour and stored in the freezer until used. All flour was from the same lot. The number of eggs needed for the entire experiment was mixed together; serving

portions of this mixture were frozen in small containers and later either baked in ramekins or made into custards as the subjects preferred. Individual servings of peaches and applesauce were prepared from large common batches and frozen before the beginning of the experiment.

Feces were eliminated in the form of ribbon-like strips and collected on cellophane. Carmine was used to indicate the beginning and the end of a period. Since the diet contained no meat or other dark-colored food, the color of the feces aided in making the separations. The total weight of the fecal excretion for the period was determined and the feces were divided into daily portions on the basis of that weight. In some cases estimation of the daily weight was difficult since the consistency of the feces varied. An acid digest of the fecal excretion for each day of the last 6 days of every 8-day period was analyzed separately. Dry-weight determinations were made on the fecal slurries by drying a 25-ml aliquot to a constant weight.

The foods and the fecal digests were evaporated to dryness in platinum evaporating dishes and ashed in a muffle furnace at 500°C. for 20 hours. The ashed samples were heated with concentrated hydrochloric acid to convert the pyrophosphate to orthophosphate. Most of the samples required a second ashing. When this was necessary a correction was made for the filter paper which was included with the incompletely ashed part of the sample during the second ashing. The iron content of the ashed samples was determined by the o-phenanthroline method of Saywell and Cunningham ('37). The color produced was measured in a photoelectric colorimeter equipped with absorption tubes which provided a light path of 50 mm through the liquid sample (Ellis and Brandt, '49).

Urine collections and fecal digests were made into two-day composites for analysis for nitrogen by a semi-micro Kjeldahl method. The nitrogen content of samples of all food was also determined.

RESULTS AND DISCUSSION

The mean iron content of the diet for all subjects for periods I, II and III was 1.03, 2.15 and 3.22 mg (table 1), respectively. When the mean intake was 1.03 mg, the amount of iron excreted in the feces was nearly the same as the intake; the mean difference was 0.04 mg. As the intake increased, the mean difference between intake and excretion

TABLE 1

Mean values for iron and nitrogen for each subject for 6-day balance periods

SUBJECTS	IRON		DRY WT. OF FECES	NITROGEN RETAINED	BODY WEIGHT	CALORIC INTAKE
	Intake	Feces				
	mg	*mg*	*gm*	*gm*	*kg*	
	Period I Nitrogen intake 9.2 ± 0.13 gm					
E.E.	1.10	1.04	17.4	— 0.8	60.9	2,520
H.H.	1.16	1.12	15.6	+ 0.2	64.1	2,690
S.J.	0.96	0.95	16.2	+ 0.6	49.1	2,110
R.L.	0.96	0.99	16.6	— 0.6	52.9	2,120
E.W.	1.06	0.89	20.6	— 1.0	48.6	2,380
C.B.	0.98	0.98	18.1	— 0.4	53.2	2,140
B.O.	0.98	0.99	13.8	— 0.3	51.3	2,120
Mean	1.03	0.99	16.9	— 0.3	54.3	2,300
	Period II Nitrogen intake 11.6 ± 0.23 gm					
E.E.	2.26	1.80	16.8	+ 0.2	60.4	2,530
H.H.	2.22	1.90	15.3	+ 0.9	64.4	2,690
S.J.	2.10	1.88	16.0	+ 0.8	49.8	2,190
R.L.	1.95	1.95	19.8	— 0.2	52.7	2,100
R.I.	2.18	1.86	23.4	+ 0.3	54.0	2,140
E.W.	2.20	2.14	24.8	— 0.6	48.6	2,380
Mean	2.15	1.92	19.3	+ 0.2	55.0	2,340
	Period III Nitrogen intake 12.8 ± 0.42 gm					
E.E.	3.21	2.34	17.7	+ 0.3	61.0	2,530
H.H.	3.28	2.70	17.7	+ 0.6	63.8	2,690
S.J.	3.21	2.48	18.5	+ 1.2	51.4	2,230
R.L.	3.21	2.74	22.6	+ 0.6	53.1	2,230
R.I.	3.26	2.78	24.5	+ 0.4	54.2	2,230
E.W.	3.19	3.22	27.4	+ 0.2	49.5	2,380
Mean	3.22	2.71	21.4	+ 0.6	55.5	2,380

increased to 0.23 mg at the 2.15-mg intake and to 0.51 mg at the 3.22-mg intake. As the intake was increased greater variability in the fecal excretion of iron occurred among the subjects.

To determine that part of the fecal-iron which was derived from gastrointestinal sources, a regression of the fecal excretion of iron on food intake was calculated. In addition to the food intakes and fecal excretions of the 8 subjects herein reported, three additional pair of food and fecal values reported by Johnston et al. ('54) for two subjects on daily intakes of 1.48 and 1.70 mg of iron, respectively, were included. The intakes of iron of these subjects were within the range of the intakes in the present study. A regression formula [1] derived by Robeson ('52) was used. This formula applies when the variance of the dependent measurement (fecal-iron) increases with an increase of that measurement and when the dependent measurement is constant at zero for the independent value (food-iron). When the regression line was extended to zero intake of iron, the calculated value for the fecal excretion of gastrointestinal iron was 0.17 mg (fig. 1). The regression was highly significant at the 0.01 level of probability.

In order that the effect of higher intakes of iron might be studied and also that the number of items might be increased, another regression was calculated, which, in addition to the figures used in the first regression, included other figures from studies previously reported from this laboratory (Johnston et al., '49; Schlaphoff and Johnston, '49; Johnston and McMillan, '52). When the regression line was extended to zero, the fecal excretion of gastrointestinal iron at zero intake of iron was 0.06 mg (fig. 1).

[1] Robeson, D. S. 1952 A supplement to the ratio of averages and the average of ratios as "best" estimates in regression. Bull. no. BU-35-M, Biometrics Unit, Cornell University.

$$b = \frac{SY - n\left(\frac{S\ \frac{Y}{X}}{S\ \frac{1}{X}}\right)}{SX - \frac{n^2}{S\ \frac{1}{X}}}$$

One might assume that the value for gastrointestinal iron in the feces at zero intake obtained from the extension of the regression line for the low intakes is more dependable because those values fall nearer to zero and the line did not have to be extended so far. Thus the indication is that the

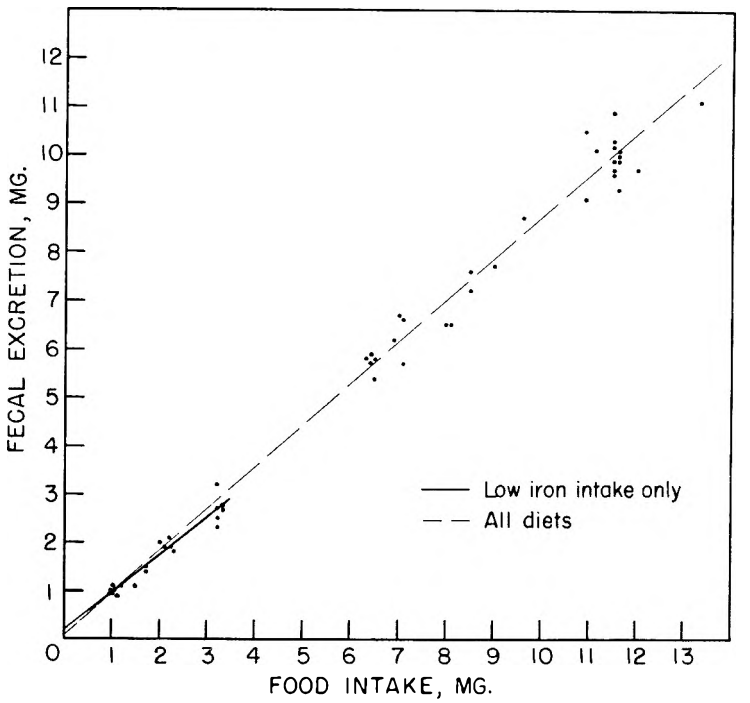

Fig. 1 Regression of iron in the feces on food-iron.

amount of iron in the feces from gastrointestinal sources falls between 0.06 and 0.17 mg, the likelihood being that the higher figure is the more accurate.

A second method for determining the amount of iron in the feces from gastrointestinal sources using the values found with the 8 subjects on the three low intakes is based on two assumptions: first, that the amount of gastrointestinal iron in the feces is the same at all intakes of iron, and second, that the percentage of absorption of iron at the extremely low level of 1, 2 or 3 mg is essentially the same. With these two assump-

tions in mind, the percentage absorption at which the gastrointestinal iron in the feces was the most constant for the three levels of intake was calculated by first determining the amount of food-iron in the feces when the percentages for absorption were varied from 10 to 25%. The subtraction of the value for the iron content of the food-residue in the feces from the total amount of fecal-iron gives the value for the gastrointestinal iron in the feces at the postulated percentages of absorption. By use of this method of computation, the absorption of iron was found to lie between 18 and 25% and the value for gastrointestinal iron in the feces between 0.12 and

TABLE 2

Calculations for gastrointestinal iron in the feces when 21% is absorbed

PERIOD	FOOD [1] IRON	IRON ABSORBED	FECAL IRON		
			Food residue	Total [1]	Gastrointestinal sources
	mg	*mg*	*mg*	*mg*	*mg*
I	1.03	0.22	0.81	0.99	0.18
II	2.15	0.45	1.70	1.92	0.22
III	3.22	0.68	2.54	2.71	0.17
Mean	2.13	0.45	1.68	1.87	0.19

[1] Analyzed value.

0.27 mg. The gastrointestinal iron was the most nearly constant for the three intakes when the percentage for absorption was assumed to be 21%. The mean amount of gastrointestinal iron in the feces at a percentage for absorption of 21% was 0.19 mg for the three intakes (table 2), a figure which agrees well with the 0.17 mg obtained by the alternative method of calculation.

In the second method for determining the amount of gastrointestinal iron, the assumption was made that the amount is the same at all intakes of iron. To justify this assumption, the sources of this iron must be examined. There are three sources of gastrointestinal iron. (1) Some may be secreted through the intestinal wall as an excretory product. This

amount was found to be negligible by McCance and Widdowson ('38) who injected iron intravenously and found no increase in the fecal excretion of iron. (2) A minute amount of iron may be a component of cells desquammated from the epithelial lining of the gastrointestinal tract. (3) Some intestinal iron originates in the digestive juices. Of the digestive juices, bile probably contributes the largest amount. Approximately 350 to 1,000 ml (Jacobi et al., '42; Gauss, '45) of bile containing 0.06 to 4 mg of iron per liter (Judd and Dry, '35) flows from the bile duct daily. Rechenberger and Pollack ('44) found 1 and 3 mg respectively of bile iron entering the intestine in two trials on men. How much iron is reabsorbed from bile is unknown although Rechenberger and Pollack found very little excreted in the feces. The iron in bile is derived from the hemolysis of red cells, a process which occurs continuously at a constant rate. Since the largest part of the gastrointestinal iron is from the bile and since the amount of iron in the bile is constant, the excretion of gastrointestinal iron is probably constant also.

The results of the two methods for calculating the amount of gastrointestinal iron in the feces yield values which are about the same. By either procedure, the daily amount approximates 0.2 mg. This is the same as the lower value of 0.2 mg reported by Dubach et al. ('49) who attributed all the radioiron in the feces from 24 to 140 days after an injection of the isotope to iron derived from hemoglobin.

If a relationship exists between the dry weight of the feces and the iron content, the fecal dry weight can be used as an aid in judging the accuracy of fecal separations. To find whether such a relationship exists, a correlation between the dry weight and the iron content of the feces was calculated for each subject for each period. The correlations from the three periods for each subject were combined by the use of Fisher's table of "z" values. Since Subjects C. B. and B. O. participated in the study for one period only and few data for these subjects were available, correlations were not calculated. The correlation coefficients for 6 subjects were: 0.93, 0.92, 0.76,

0.74, 0.51 and 0.26. These correlations are significant at 0.01 level of probability except for Subjects H. H. (0.51) and S. J. (0.26). Some of the subjects, including H. H. and S. J., were given mineral oil. Before calculation of the correlations, the weight of the mineral oil was subtracted from the dry weight of the fecal excretion corresponding to the day that the mineral oil was given. Perhaps these two subjects retained the feces so long that the oil spread throughout the residue of the food for several days making the subtraction of the weight of the oil from the excretion for a single day inaccurate.

The mean protein intake, as calculated[2] from the nitrogen values (table 1), was 59, 74 and 81 gm for periods I, II and III respectively. The amount of nitrogen retained by each subject was determined to aid in evaluation of the adequacy of the diet. Although 59 gm of the protein in all three diets was from milk, this amount of high quality protein did not assure a positive nitrogen retention. The amount retained appeared to depend upon the previous dietary intake. During the first period two of the 7 subjects retained nitrogen. These two subjects (S. J. and H. H.) were accustomed to an intake of from approximately 50 to 56 gm of protein daily according to a 7-day dietary record taken preceding the experimental period. On a per kilogram basis this was lower than the customary protein intake of the other subjects. During the second period, Subjects R. L. and E. W., the only ones who lost nitrogen, had been consuming a daily average of 70 and 96 gm protein respectively on self-selected diets, a higher intake per kilogram of body weight than the other subjects. During period III all subjects were retaining nitrogen. Difference in the amount of nitrogen retained was probably not due to differences in the adequacy of the caloric intake. The individual caloric needs were determined from charts of height and body build (Metropolitan Life Insurance Co., '42). The caloric intake was apparently adequate since there was little change in the weight of the subjects during any experimental period.

[2] Protein in milk = 6.38 × nitrogen; protein in the other foods = 6.25 × nitrogen.

The caloric intake for Subject E. W. was high for an individual of her size and yet she gained no weight during the experimental period. The authors suspected that her basal metabolic rate was abnormally high. A year later, a determination of her basal metabolic rate was made and although it was somewhat high, it was within the normal range. She excreted more fecal iron in periods II and III and more fecal nitrogen in all periods than the other subjects. The dry weight of her feces was higher than that of any other subject.

SUMMARY

Young women were given diets, the mean content of which was 1.03, 2.15 and 3.22 mg of iron during three 8-day periods respectively. The iron in the food and feces and the nitrogen in the food, feces and urine were determined.

The amount of iron in the feces from gastrointestinal sources was calculated by two methods. (1) A regression of the values for fecal-iron on food-iron was extended to zero. The calculated value for fecal-iron at zero intake was 0.17 mg. (2) The percentage absorption at which the gastrointestinal iron in the feces appeared to be the most constant for the three levels of intake was determined. By this calculation, the absorption for the three intakes lay between 18 and 25% and the value for gastrointestinal iron lay between 0.12 and 0.27 mg. By either method of calculation, the mean value for daily gastrointestinal iron in the feces approximated 0.2 mg.

To determine the relationship between the dry weight and the iron content of the feces, a correlation between dry fecal-weight and fecal-iron was calculated for each subject. The correlations were highly significant except for two subjects who had been given mineral oil and whose fecal excretions were delayed more than for the other subjects.

The amount of nitrogen retained by each subject on the three diets was determined to aid in the evaluation of the adequacy of the diet. The mean protein intake for the three periods was 59, 74 and 81 gm respectively. Although most of the protein was from milk, some subjects did not retain nitro-

gen during the first two periods. The amount of nitrogen retained by an individual reflected the protein intake of that person previous to the experimental period.

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THE UTILIZATION OF PANTETHINE AS COMPARED TO CALCIUM PANTOTHENATE BY THE CHICK

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THREE FIGURES

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The characterization of the *Lactobacillus bulgaricus* factor (LBF) as N-(+)-pantotheny1-2-aminoethanethiol (pantetheine) or the disulfide (pantethine) and the synthesis of pantethine were reported by Snell et al. ('50). The details of the synthesis and purification of pantethine and pantetheine have been published by Baddiley and Thain ('52), King et al. ('53), Schwyzer ('52), Viscontini et al. ('53) and Wittle et al. ('53).

McRorie et al. ('50a) and Brown et al. ('50) found that the LBF factor was interchangeable with pantothenate in the nutrition of *Lactobacillus casei* and *Lactobacillus arabinosus*. The growth promoting properties of pantethine and a natural occurring form of LBF (LBF-1A) were described for 22 microorganisms by Craig and Snell ('51).

Few reports have appeared concerning the biological activity of pantethine as compared with pantothenate in animals. Rasmussen et al. ('50) have shown that a concentrate of LBF which was free of vitamin B_{12} enhanced the growth of chicks fed a diet either free of animal protein or supplemented with fish solubles. McRorie et al. ('50b), in a comparison of the growth obtained by supplementing pantothenic acid-deficient rats with either calcium pantothenate or an LBF concentrate, demonstrated that LBF could replace panto-

thenic acid in the nutrition of the rat. Lih et al. ('51) found that coenzyme A and an LBF concentrate (LBF-1A) were equal to pantothenate in promoting growth in the rat.

Because of the occurrence of the *Lactobacillus bulgaricus* factor in nature and the importance of pantothenic acid to the nutrition of higher animals, it seemed of interest to determine the relationship between highly purified preparations of pantethine and calcium pantothenate in promoting chick growth.

EXPERIMENTAL

Day-old S. C. White Leghorn chicks of mixed sexes obtained from a commercial hatchery, were housed in electrically-heated batteries with raised screen floors. The following procedure, similar to that of Jukes ('47), was followed in preparing the chicks for the pantethine-pantothenic acid assay. The chicks, other than those used in test 5 which received a commercial broiler ration for only three days prior to the depletion period, received ad libitum a diet of the following percentage composition for 7 days: ground yellow corn 63.2, solvent extracted soybean meal 28.0, corn gluten meal 2.5, alfalfa leaf meal 2.0, steamed bone meal 3.0, calcium carbonate 0.5, iodized salt 0.5, choline chloride 0.05, DL methionine 0.05, and manganese sulfate 0.03. In addition, the following were added in milligrams per kilogram of diet: riboflavin 3.5, niacin 22, calcium pantothenate 10, vitamin B_{12} 0.02, and potassium iodide 2. The addition to the diet of a vitamin A and D concentrate supplied 6000 U.S.P. units of vitamin A and 2400 of vitamin D per kilogram.

Following this preliminary growing period, the diet of the chicks in each test was replaced by a purified diet deficient in pantothenic acid. The composition of this diet is presented in table 1. A supplement of crystalline vitamins (other than calcium pantothenate) diluted with purified casein was added to the diet at a level of 1% as shown in table 1. This supplement furnished the following amounts of vitamins in milligrams per 100 gm of diet: menadione 0.05, thiamine 0.4, riboflavin 0.8, pyridoxine 0.6, niacin 2.0, *p*-aminobenzoic acid 15.0,

inositol 50.0, biotin 0.02, vitamin B_{12} 0.002 and pteroylglutamic acid 0.2. The purified diet was stored under refrigeration at 5°C. until fed to the chicks. During these assays the water pans were scrubbed and refilled daily with fresh tap water. The chicks were fed ad libitum the pantothenic acid-deficient diet until a decline occurred in the daily rate of gain. At the end of an 8-to 14-day depletion period, the lightest and heaviest birds were discarded (about 10% of the total number), and the remaining chicks were distributed in the assay in such a

TABLE 1

Composition of the pantothenic acid-deficient diet

INGREDIENT	AMOUNT
	%
Casein (hot alcohol extracted)	24.1
Corn starch	51.9
Gelatin	10.0
Cell-u-flour	3.0
Salts (Jones and Foster, '42)	5.0
Lard	4.0
Vitamin mix	1.0
Vitamins A and D oil (4000 U.S.P. units A and 400 D/gm	0.4
L-Cystine	0.3
Choline chloride	0.2
Manganese sulfate	0.1
Vitamin E (35% α-tocopherylacetate)	0.024

manner as to obtain equal distribution of body weight among the respective groups. Although the number of chicks assigned to a group in an assay varied from 10 to 12 in the experiments to be reported, the number of birds per group in any specific assay was kept uniform. Since survival of chicks during the assay was excellent, survival data have not been included in the tables that follow. Graded doses of calcium pantothenate and the compounds to be assayed for pantothenic acid activity were either mixed in the purified diet, administered by pipette, or injected intraperitoneally. Pipetting and injecting of supplements was performed three times weekly.

The chicks were weighed weekly during the14- or 20-day assay period.

RESULTS AND DISCUSSION

Incorporation of pantethine in the diet

The data obtained from those assays in which three different preparations of pantethine were incorporated in the diet are presented graphically in figures 1, 2, and 3. Equations

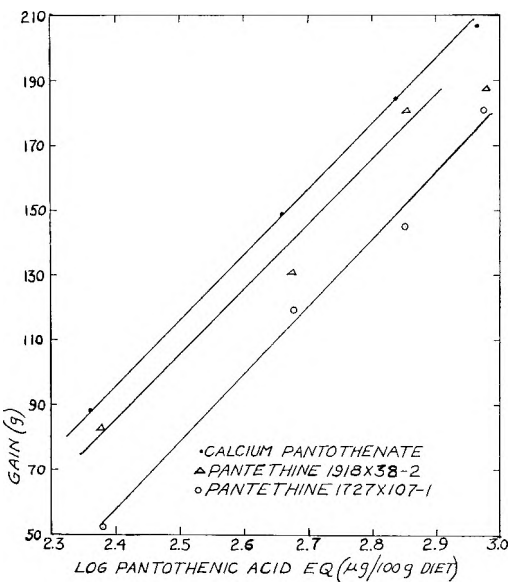

Fig. 1 Weight gains in test 1 by chicks receiving either calcium pantothenate or pantethine in the diet. Equations for the regression lines are as follows: •——• $y = 203\ (x) - 393$; Δ——Δ $y = 200.8\ (x) - 397$; o——o $y = 208\ (x) - 442$; where y is gain and x is log dose.

for the straight lines relating growth response to the log dose of the pantothenic acid equivalent were calculated by the method of least squares as described by Bliss ('51). It is to be noted that the response to pantethine supplementation as measured by growth is linear and essentially parallels the response curve of the standard. Only in test 3 (fig. 3) was a response obtained with pantethine that was equal to the response of the standard.

The purity of the pantethine preparations as determined microbiologically, the percentage of the theoretical content of pantothenic acid in these pantethine preparations as found

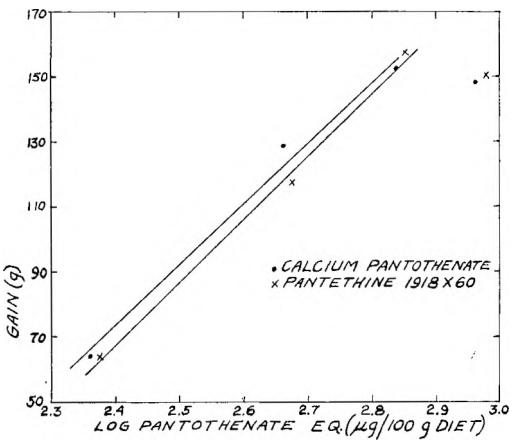

Fig. 2 Weight gains in test 2 by chicks receiving either calcium pantothenate or pantethine in the diet. Equations for the regression lines are as follows: •——• $y = 187.5\ (x) - 377$; x——x $y = 193.2\ (x) - 396$; where y is gain and x is log dose.

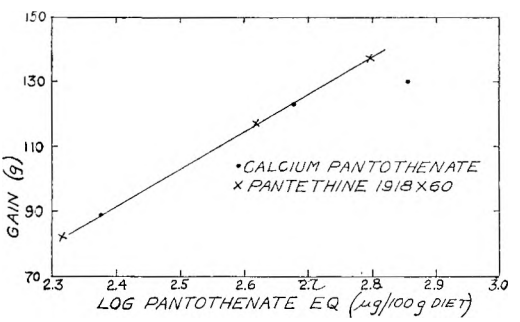

Fig. 3 Weight gains in test 3 by chicks receiving either calcium pantothenate or pantethine in the diet. Equations for the regression lines are as follows: •——• $y = 113.3\ (x) - 180$; x——x $y = 115.4\ (x) - 185$; where y is gain and x is log dose.

by chick assay, and the relative potency of pantethine as compared to the standard, calcium pantothenate, are presented in table 2. These data indicate that pantethine when incorporated in the diet is at least 90% as active as calcium panto-

thenate on an equivalent basis in promoting chick growth. This loss of about 10% in potency might be due to any one or several of the following factors: destruction of pantethine in the gastro-intestinal tract, greater utilization of pantethine by the intestinal flora as compared to pantothenate, or decreased absorption of pantethine from the intestinal tract. It does not appear likely that this loss of potency was due to destruction of pantethine in the diet since the compound is quite stable under the conditions employed.

TABLE 2

Relative potency of pantethine incorporated in the diet as compared to calcium pantothenate on an equivalent basis

PANTETHINE PREPARATION	PURITY BY MICROBIO. ASSAY [1]	CHICK ASSAY [2]	
		$\frac{\text{Pantothenic acid found}}{\text{Pantothenic acid calc.}} \times 100$	Relative potency
	%		%
1727X107-1	76	67.1	88.4
1918X38-2	100	88.3	88.4
1918X60	100	94.4	94.4
		100.0	100.0

[1] Procedure of Craig and Snell ('51).

[2] Calculation by method of Bliss ('51).

Administration of pantethine by pipette

The data as presented in test 3 (table 3) show that the growth response obtained at the lower level of pantethine (44 μg) which was administered by mouth corresponds to the response obtained with a similar amount of pantothenate. However, at an increased level of pantethine administration (88 μg) the response is considerably less than that which would be expected. This atypical response to a higher dose of the compound was observed in several assays in which pantethine was given by mouth three times weekly.

The failure of the chicks to respond to dosages of orally administered pantethine which should have supplied them with an optimal amount of pantothenic acid activity was

further studied, and the data obtained are presented in test 4 (table 3). In addition to the administration of graded doses of calcium pantothenate and pantethine by pipette three times weekly, pantethine was administered at a level of 100 μg per day in a divided dose which was given in the

TABLE 3

Weight gains by chicks following administration of calcium pantothenate or pantethine by pipette three times weekly

SUPPLEMENT	DOSAGE	PANTOTHENIC ACID EQ.	WEIGHT GAIN
	μg/day	*μg/day*	*gm*
	Test no. 3 — 14 days		
None	...	...	40
Calcium pantothenate	42.9	39.4	99
	85.8	79.0	153
Pantethine (1918X60)	44.0	34.8	103
	88.0	69.6	112
	Test no. 4 — 14 days		
None	...	...	15
Calcium pantothenate	21.5	19.8	61
	42.9	39.4	104
	85.8	79.0	152
Calcium pantothenate in diet 1.5 mg/100 gm	...	...	163
Pantethine (1918X60)	25	19.8	56
	50	39.6	90
	100	79.2	114
	200	158.4	161
Pantethine divided into A.M. and P.M. dosage	100	79.2	138

morning and afternoon on the three respective treatment days during the week. As previously found, pantethine failed to produce a maximum growth response at the anticipated dosage level (about 100 μg). However, a maximum response occurred at a level of 200 μg of pantethine per day (or the equivalent of 158 μg of pantothenic acid) which is twice the

equivalent amount of calcium pantothenate required to obtain maximum chick growth.

It is interesting to note that the administration of pantethine in the morning and afternoon of the three treatment days per week produced a growth response that was to be expected on the basis of the responses obtained at the lower dosages of pantethine. The results of this test would indicate that either a defect exists in the absorption of pantethine or, if pantethine is cleaved prior to absorption, this cleavage is inefficient. However, more efficient utilization occurs if pantethine is incorporated in the diet or given by mouth in relatively small and more frequent doses.

Intraperitoneal injection of pantethine

The results of three tests as presented in table 4 indicate that pantethine is equally as active as calcium pantothenate on an equivalent basis in promoting chick growth when injected intraperitoneally.

Results similar to those presented here have been reported for another compound containing pantethine as one of its components. Hegsted and Lipman ('48) found that an average of only 61% of the theoretical pantothenic acid content of coenzyme A was utilized if the preparation was administered by pipette to chicks. However, if injected, coenzyme A was equally as active as an equivalent amount of calcium pantothenate. These authors indicated that a failure in absorption of ingested coenzyme A would explain its apparent decrease in relative potency following oral admiistration. Subsequently, Govier and Gibbons ('51) concluded that in the dog orally administered coenzyme A is not absorbed *per se,* but is apparently broken down to produce a compound with a structure simpler than that of pantetheine.

Pantetheine, the reduced form of pantethine, and the mercury mercaptide of pantetheine (Wittle et al., '53) have also been administered to chicks, both by pipette and by intraperitoneal injection. It was possible to maintain pantetheine in

the reduced state during the assay by keeping a butanol solution of this compound under nitrogen. Prior to each treatment an aliquot of the butanol solution was diluted with water to the required concentration and immediately administered to the chicks. There were no significant differences in the results obtained with pantethine, pantetheine, or the mercury mercaptide of pantetheine.

TABLE 4

Weight gains by chicks following intraperitoneal injection of calcium pantothenate or pantethine three times weekly

SUPPLEMENT	DOSAGE	PANTOTHENIC ACID EQ.	WEIGHT GAIN
	μg/day	*μg*	*gm*
	Test no. 2 — 14 days		
None	...	...	34
Calcium pantothenate	25.6	23.6	52
Pantethine (1918X60)	30.0	23.8	56
	Test no. 3 — 14 days		
None	...	...	40
Calcium pantothenate	42.9	39.6	100
	85.8	79.1	146
Pantethine (1918X60)	44.0	34.8	112
	88.0	69.6	131
	Test no. 5 — 14 days		
None	...	...	11
Calcium pantothenate	43.6	40.0	54
	87.2	80.0	98
Pantethine (1918X60)	50.6	40.0	49
	101.2	80.0	98

SUMMARY

1. Pantethine was compared with calcium pantothenate in chick growth assays by three methods of administration: incorporation in the diet, by mouth, and intraperitoneal injection.

2. Pantethine when incorporated in the diet was found to be at least 90% as active in promoting chick growth as

calcium pantothenate based on the equivalent pantothenic acid content.

3. The relative potency of pantethine administered by pipette was similar to that found when the compound was incorporated in the diet if the dosages administered were relatively small. However, at higher dosage levels, pantethine was considerably inferior to calcium pantothenate.

4. Pantethine was equally as active in promoting growth as calcium pantothenate when injected.

5. Pantetheine or its mercury mercaptide were as active as pantethine when administered by mouth or intraperitoneally.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. E. L. Wittle of Parke, Davis and Company for the preparations of pantethine, pantetheine, and the mercury mercaptide of pantetheine; and to Mrs. V. M. McGlohon for the pantethine microbiological assays.

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CHOLINE DEFICIENCY IN THE RABBIT[1]

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SEVEN FIGURES

(Received for publication February 11, 1954)

Information on the choline requirements of the rabbit is scanty. Although hepatic cirrhosis has been produced by diet variations (Spellberg, Keeton and Ginsberg, '42; Rich and Hamilton, '40), the cause of the syndrome was not established and choline was not directly implicated. Very probably the cirrhosis observed did result from inadequate dietary choline, and this has been assumed by some reviewers on the subject. Blumberg, MacKenzie and Seligson ('42) reported, in abstract, that cirrhosis of the liver in the rabbit was preventable by choline.

It is the purpose of this paper to report the production of severe choline deficiency in the rabbit and to describe the symptoms produced.

EXPERIMENTAL

Weanling 4-week old rabbits of the New Zealand White and the California White strains were placed immediately on the purified diets. The choline-deficient basal diet was made up to contain in per cent, 30 methanol-extracted peanut meal, 6 purified casein, 4 salt mixture no. 5, 29.9 sucrose, 0.1 cystine,

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10 Ruffex,[2] 19 lard, and 1 cod liver oil. Vitamins were added to give the following levels per gram of diet: thiamine, riboflavin, pyridoxine, 3 μg each; calcium pantothenate, 17 μg; *i*-inositol, 200 μg; niacin, 20 μg; methyl, 1-4, naphthoquinone, 0.3 μg. The diet is the same as used in other choline deficiency studies in this laboratory (Schaeffer, Copeland and Salmon, '51), except for the addition of Ruffex. The residual choline content of the diet was established as 0.006%.

Additions to the basal diet of choline, methionine, vitamin B_{12} plus folacin, ethanolamine and monomethylethanolamine were made at levels indicated. Vitamin E was given as DL, α-tocopheryl acetate solution in olive oil by dropper, orally, at a level finally set at 10 mg daily. This is fully 10 times the normal requirement for rabbits (Hove and Harris, '47). The reason for this high level is discussed in a subsequent paper (Hove and Copeland, '54).

Blood samples were drawn at intervals by heart puncture. Hemoglobin, icteric index, plasma bile pigments (Van den Berg) and urinary urobilinogen and porphobilinogen (Ehrlich's test) were determined as indicated in standard texts.

RESULTS

Effect of choline on growth

The basal diet without added choline did not support growth in rabbits (table 1 and fig. 1). Weanling rabbits, started on the diet at body weights of 500 to 1000 gm, gained slightly then leveled off or declined somewhat, and thereafter maintained a fairly constant body weight for long periods. There was some hair loss, and in one case complete denuding. No diarrhea occurred. None of the rabbits had respiratory infections. Death ensued with little or no premortal weight loss.

Adult animals had a much more critical need for choline than did weanling rabbits (fig. 2). An immediate weight loss occurred when choline was removed from the diet of two rab-

[2] Ruffex (Fisher Scientific Co.) is processed rice bran.

TABLE 1

Body weight and liver damage in choline deficient rabbits

RABBIT NO.	BODY WEIGHT AT DAYS			DEATH		LIVER DAMAGE	
	0	50	100	Days	Body weight	Nodular cirrhosis	Fat (dry basis)
	gm	*gm*	*gm*		*gm*		*%*
85	530	790	980	161 [1]	1080	Moderate	38.3
81	620	730	910	181	930	Moderate	53.6
84	660	990	960	231	960	Severe	45.0
78 [2]	1180	1100	1120	102	1040	Moderate	58.2
80	1290	960	....	66	968	Moderate	46.9
8	1860	1990	1610	217	1420	Severe	39.7
79 [3]	1060	2140	2540	....	....		
70 [3]	420	1580	2150	....	....	None	13.8
71 [3]	770	1830	2590	....	....	None	12.3

[1] Killed accidentally.

[2] Vitamin B_{12} (40 μg/kg) and folacin (2 mg/kg) added to basal diet.

[3] Choline chloride added at 0.12% to the basal diet.

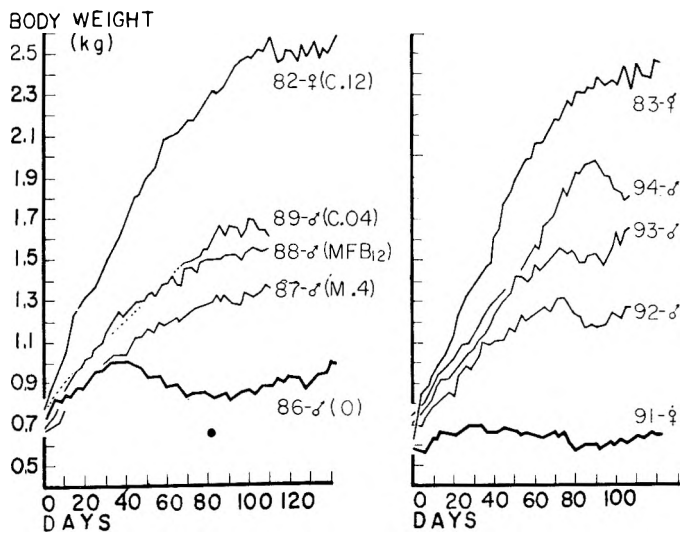


Fig. 1 Growth rates of rabbits on the choline-low basal diet (86, 91), or this diet supplemented with 0.4% DL Methionine (87, 92), methionine plus 40 μg vitamin B_{12} and 2 mg folacin per kilogram (88, 93), or 0.04% choline Cl (89, 94), or 0.12% choline Cl (82, 83).

bits maintained on the basal diet with added choline for 170 days, and weighing about 3 kg. These animals died after 45 and 53 days with severely cirrhotic and fatty livers. Two similar animals continued on the choline-supplemented diet were completely normal when killed after 53 days. Four other rabbits, approximately two-thirds grown (2.28 kg),

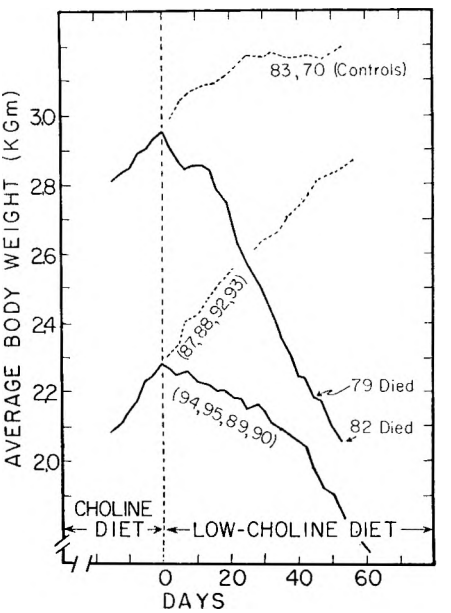


Fig. 2 Acute and immediate body weight loss due to the removal of choline from the diet of adult rabbits. The livers of rabbits 79 and 82 were grossly cirrhotic, and had an average fat content of 53.4% dry basis, while those of 83 and 70 were normal with an average fat of 11.2%.

were transferred from the choline-supplemented to the low-choline diet. The weight loss again began immediately but not so disastrously (fig. 2).

The basal diet supplemented with 0.12% choline chloride allowed excellent growth in rabbits (about 25 gm/day during 50 days from weaning). Increase of the choline level to 0.20% did not further increase the growth rate.

From figure 1 it is evident that the addition of 0.04% choline chloride to the diet was insufficient to meet the needs of

the growing rabbit. DL-Methionine added to 0.4% was less effective than the 0.04% choline, although some protective action of methionine was evident.

Vitamin B_{12} (40 μg/kg) plus folacin (2 mg/kg) added to the basal diet had no beneficial effect on the choline-deficient rabbit no. 78 (table 1). Similarly, this supplement did not improve the response to 0.04% choline (fig. 3).

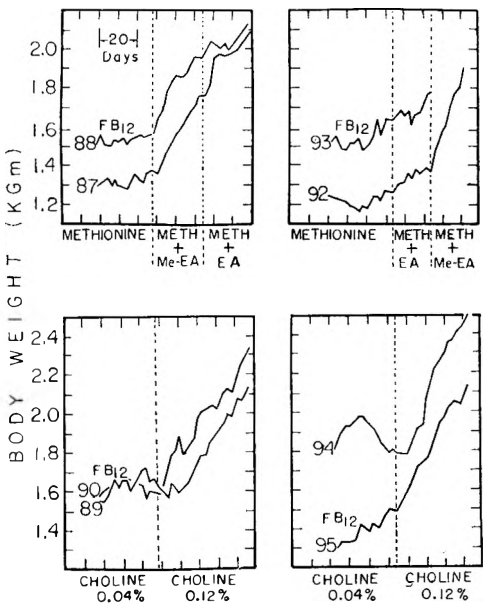


Fig. 3 Stimulation of rabbit growth by monomethylethanolamine (Me-EA), but not by ethanolamine (EA), added at 0.2% to the choline-low basal diet containing 0.4% of DL-Methionine. The symbols (F B_{12}) indicate the addition of 40 μg vitamin B_{12} and 2 mg folacin per kilogram of diet throughout the experiment.

Although the vitamin B_{12}-plus-folacin supplement did not spare the low level of choline, it appeared to improve the utilization of methionine (fig. 1).

Monomethylaminoethanol added at 0.2% to the diet with 0.4% methionine, permitted a prompt and strong growth response (fig. 3). Folacin plus vitamin B_{12} appeared not to be necessary for this effect. On the other hand, 0.2% ethanola-

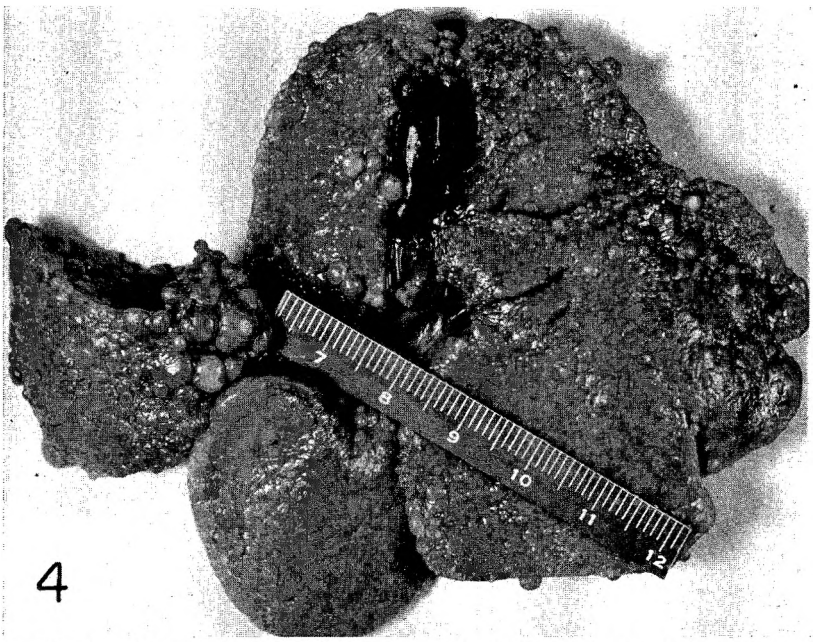
mine similarly added to the diet did not result in a growth response (fig. 3).

Liver and kidney damage in choline-deficient rabbits

Nodular cirrhosis of the liver of rabbits occurred readily as a result of choline deprivation, and the livers were consistently fatty (38.3 to 58.2% fat, dry basis). To date 18 rabbits have died from choline deficiency and all have had grossly evident cirrhosis. The earliest development was 45 days in rabbit 79, but the most severe was in rabbit 84 which died after 213 days. Ascites and occasional hydrothorax were noted. The younger the rabbit when started on the choline-deficient diet, the longer it lived and the more severe was the cirrhotic condition of the liver. With adequate dietary choline, liver cirrhosis has never been noted in about 70 animals from other experiments, as well as controls in the present experiments. Liver fat of normal rabbits averages 11.8%, dry basis.

The cirrhotic livers were a pale tawny color in gross appearance. In the most severe cases the entire surface of the liver was covered with nodules varying from 1 to 5 mm in diameter. The nodules varied in color from pale yellow to red. The gross appearance of one of the livers is illustrated in figure 4. Microscopic studies of slides prepared from the liver revealed an advanced stage of nodular cirrhosis. The normal architecture of the liver was completely lost. Pseudolobules were walled-off by bands of connective tissue (fig. 5). Slides stained with Mallory's aniline blue stain showed that there were extensive accumulations of collagenous tissue in these bands. There was considerable variation in the amount of fat in the various lobules. Extensive bile duct proliferation was observed in the livers with severe cirrhosis. Minimum amounts of ceroid were observed.

Although kidney hemorrhage has not been noted, about half of the rabbits that died from choline deficiency have had damaged kidneys. They were pale and mottled in color, and microscopic studies of sections revealed that there was exten-



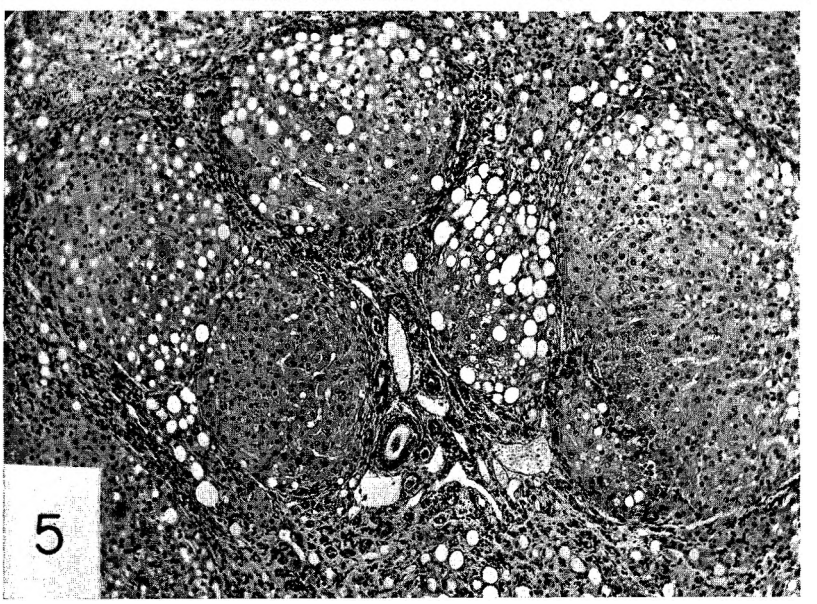


Fig. 4 Photograph illustrating gross appearance of one of the cirrhotic livers. Rabbit 84, 231 days on deficient diet.

Fig. 5 Photomicrograph of a section of liver shown in figure 4. Hematoxylin and Eosin stain, × 95.

sive tubular necrosis, particularly of collecting tubules, with atypical tubular repair. The extent of this damage is illustrated in figures 6 and 7. The regeneration of tubules has resulted in an atypical proliferation of cells within the lumen of the rebuilt tubules. Remnants of necrotic tubules are shown in the photomicrographs.

Choline effect on hematopoiesis

As early as the 70th day of the deficiency the blood plasma of the rabbits was a bright golden yellow color. Even after a 1:10 dilution the plasma was yellower than that of control rabbits; this is tabulated as icterus index in table 2. There was little or no jaundice at this time or at post mortem examination. In spite of the high icterus, the plasma was completely negative to the direct or indirect Van den Berg test for bile pigments, until much later in the deficiency when slightly positive tests were obtained. Tests on the urine showed a very highly positive reaction to the Ehrlich reagent (*p*-dimethylaminobenzaldehyde in 15% HCl). For numerical expression a ratio was used between the L_{540} of the Ehrlich test and the L_{540} of creatinine determination. The urine dilution was 1:5 in the first case, and 1:500 in the second.

The Ehrlich reaction is usually considered a test for urobilinogen. However, this compound is ether-extractable, while the monopyrollic substance porphobilinogen, which also gives the Ehrlich test, cannot be extracted by ether. Since the chromogenic substance in the urine of choline-deficient rabbits was not ether-extractable, it has been assumed to be porphobilinogen (Lemberg and Legge, '49). Further evidence for porphobilinogen was obtained by paper chromatography. Using the technique of Granick and Bogorad ('53) the red material formed with Ehrlich's reagent in rabbit urine had an R_f of 0.59; this corresponded fairly well with the R_f of 0.56 for pure porphobilinogen as given by Granick and Bogorad. The Ehrlich reagent is not very specific. Development of chromatographed rabbit urine with the Ehrlich reagent gave, not only the brick-red porphobilinogen band

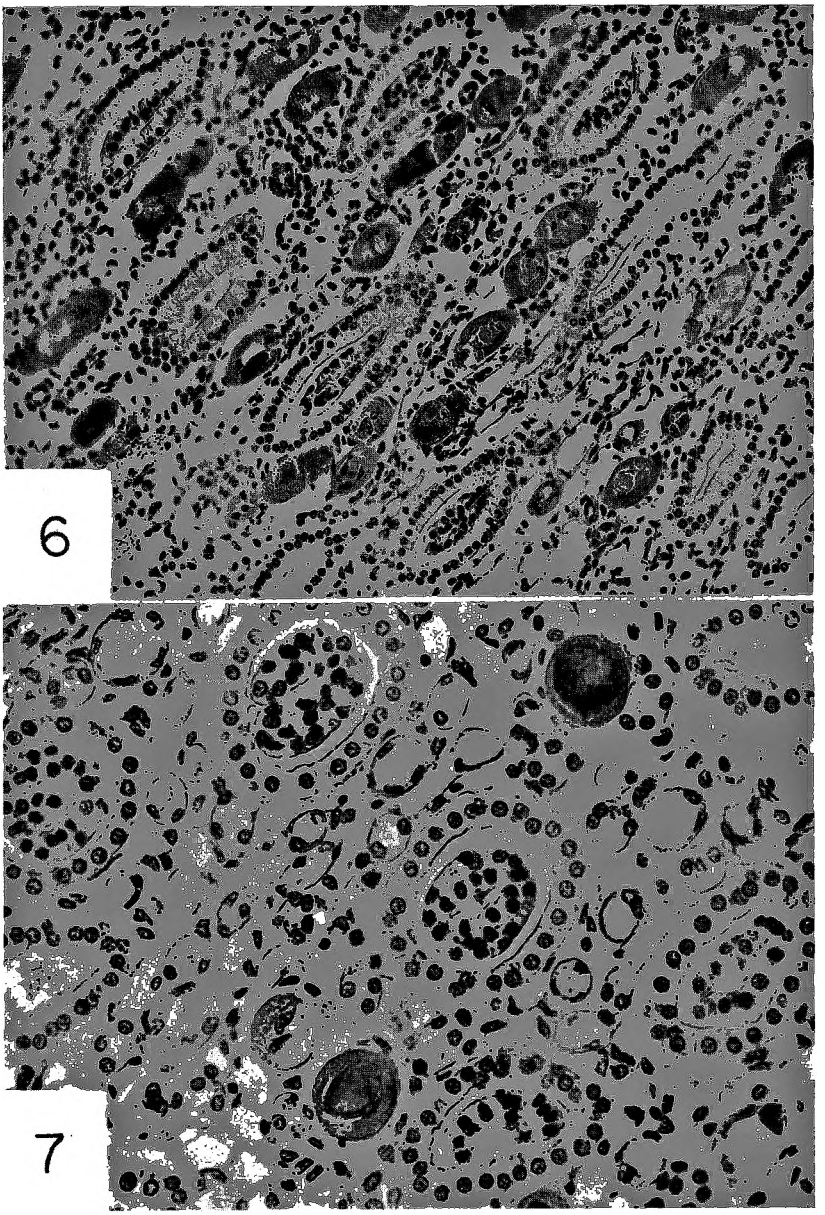


Fig. 6 Photomicrograph of a section of kidney illustrating tubular necrosis and atypical tubular repair. Hematoxylin and Eosin, × 195.

Fig. 7 Photomicrograph of a section of kidney. Higher magnification to illustrate cell structure in regenerated tubules. Hematoxylin and Eosin, × 385.

(R_f, 0.59), but a brilliant yellow broad band ((R_f, 0.48) in all urines; a weaker (allantoin) yellow band (R_f, 0.24); and a deep blueish violet band (R_f, 0.85).

A mild anemia developed in the choline-deficient rabbits. This was partially prevented by methionine additions or suboptimum choline levels. Vitamin B_{12} plus folacin supplements did not improve hematopoiesis (table 2).

TABLE 2

Blood hemoglobin and porphobilinogen excretion in choline deficient rabbits

ADDITIONS TO CHOLINE-LOW DIET	RABBIT NO.	DAYS ON DIET	ICTERIC INDEX	PLASMA BILE PIGMENTS	PORPHOBILINOGEN EXCRETION [1] (Ehrlich's reaction)	HEMOGLOBIN
						%
None	91	70	high	±	5.4	7.59
None	86	70	none	0	0.8	8.26
		90	trace	0	1.3	8.63
None	81	120	high	0	2.1	12.84
		160	high	+	4.0	10.50
None	84	120	high	0	5.2	11.71
		160	high	0	3.0	8.60
None	85	120	high	0	1.7	10.66
		160	high	+	2.4	9.57
					Average:	9.82
Choline, .12%	79	120	none	0	0.35	12.76
		160	none	0	0.17	12.75
Choline, .12%	82	120	none	0	0.39	12.41
		160	none	0	0.33	13.03
Choline, .12%	83	120	none	0	0.73	13.89
		160	none	0	0.55	12.40
					Average:	12.87
Choline, .04% [2]		70–90	none			11.32
Choline, .04%, B_{12}, Fol.[2]		70–90	none			11.00
Methionine, .4% [2]		70–90	none			11.18
Meth., .4%, B_{12}, Fol.[2]		70–90	none			11.25

[1] Expressed as ratio: (540) L (Ehrlich reaction on 1: 5 urine) to (520) L for creatinine-picrate on 1: 500 urine dilution.

[2] Averages of 4 determinations.

DISCUSSION

Dietary choline is critically required by rabbits for growth and maintenance of body weight, as well as for the prevention of fatty livers and severe hepatic cirrhosis. For the diet used, the choline requirement may conservatively be set at 0.13% of the diet (including the 0.006% residual in the basal diet). More than this gave no greater growth rate, while a total level of 0.05% was decidedly suboptimum. Adult rabbits lost weight immediately and died quickly when choline was omitted from their diet.

Methionine had a slight, although definite, effect in replacing choline. A level of 0.4% methionine gave only about half the growth stimulation obtained with 0.04% of added choline chloride. Ethanolamine, combined with methionine, was of no additional benefit. On the other hand, monomethylethanolamine with methionine gave an immediate and dramatic growth stimulation. In this respect the rabbit differs sharply from the rat (Strength, Schaefer and Salmon, '51), but more nearly resembles chicks (Schaefer, Salmon and Strength, '51).

Folacin plus vitamin B_{12} allowed the more effective use of methionine, but appeared to be unnecessary for the methylation of monomethylaminoethanol by methionine to form choline, *in vivo*. These vitamins did not improve the utilization of suboptimal levels of choline for growth.

Jaundice has not been prominently noted in the choline-deficient rabbits. The high icteric index which appeared relatively early in the deficiency was not due to plasma bile pigments, either directly or indirectly, although these do appear as the deficiency progresses. Interestingly, the deficient rabbits excreted in their urine an Ehrlich-positive material which seemingly was porphobilinogen. This is a mono-pyrrole said to be an intermediate in the synthesis of the hemoglobin molecule. The animals were moderately anemic. The high icteric index may have been due in part to porphyrin-type substances, resulting from an overflow of intermediate compounds in the incomplete hematopoietic process.

SUMMARY

Rabbits required about 0.13% choline in the diet used for growth, maintenance of body weight, and prevention of fatty and cirrhotic livers and necrosis of kidney tubules. For growth, methionine had a slight, though definite, replacement value for choline. Monomethylaminoethanol, with methionine, was a very effective substitute for choline, while ethanolamine was inactive.

Vitamin B_{12} plus folacin improved the utilization of methionine by the choline deficient rabbit for growth but appeared to be of no value in sparing suboptimum levels of choline, or in promoting the utilization of monomethylaminoethanol plus methionine.

Moderate anemia, a high icteric index but without plasma bile pigments or jaundice, and the excretion of porphobilinogen, were noted in the choline-deficient rabbits.

ACKNOWLEDGEMENT

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PROGRESSIVE MUSCULAR DYSTROPHY IN RABBITS AS A RESULT OF CHRONIC CHOLINE DEFICIENCY [1]

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SIX FIGURES

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Muscular dystrophy in man and animals is a disorder involving atrophy, degeneration and ultimate loss of function of muscle cells. The striated musculature of the limbs is usually affected first. In man the disorder occurs with moderate frequency and progresses slowly but invariably to a fatal outcome. In rabbits and other animals a dystrophy, with clinical and biochemical pathology almost identical with that occurring in man, results from a simple deprivation of dietary vitamin E. However, vitamin E therapy appears not to alter the course of the disease in humans (Milhorat, '51); nor is any other effective therapy known.

Possibly the dystrophies result from a functional interruption of nerve impulse transmission to the musculature due to a disorder in acetylcholine formation or utilization. Some years ago Torda and Wolff ('45) reported that vitamin E was a specific activator for the biological synthesis of acetylcholine from choline and acetate. Recent work has shown that coenzyme A, necessary for the acetylation of choline, contains a sulfhydryl grouping which must be in the reduced

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form for performing its function. Conceivably the fatty peroxides arising in the body of an animal deficient in vitamin E (Dam, '49) may oxidize coenzyme A to the disulfide state, thus diminishing acetylcholine formation and lessening the effectiveness of nerve impulse transmissions, whether to the striated musculature itself or to the arteries supplying blood to the muscles. An explanation is thus at hand for interpreting the dystrophy of vitamin E deficiency as an acetylcholine-mediated mechanism.

If diminished acetylcholine formation is central to the pathogenesis of the dystrophies, then a nutritional deficiency of choline ought to result in muscular dystrophy, since choline is the precursor of acetylcholine. In a like manner, deficiencies of any of the nutritional factors involved in coenzyme A formation (e.g., pantothenic acid) should also result in muscular weakness or dystrophic paralysis.

The present paper describes a muscular dystrophy in rabbits due specifically to a chronic choline deficiency.

EXPERIMENTAL

The composition of the diets is given in table 1. The rabbits used in the choline-deficiency experiments are the same as described in the previous paper (Hove, Copeland and Salmon, '54). Twenty-four-hour urine samples were collected three times weekly (Mon., Wed., Fri.). Urinary creatine (diacetyl method) and creatinine (picrate method) were determined as previously described (Hove, '52a). Blood for plasma vitamin E was drawn by heart puncture. The vitamin E was extracted by the Kimble ('39) procedure and determined by the Emmerie-Engel reaction in alcohol at a one-minute time. Vitamin E was calculated as the total fat-soluble reducing substance in plasma, expressed as α-tocopherol.

RESULTS

Muscular dystrophy due to choline deficiency

Criteria of muscle dystrophy in rabbits were: creatinurea, diminished urinary creatinine, and muscular weakness, espe-

cially in the hind legs. A convenient gauge of muscular weakness is obtained by allowing the rabbit hind legs to hang over the edge of the cage. A moderately dystrophic rabbit struggles to kick itself back into the cage but cannot. A normal rabbit of the same age or weight bounds into its cage with one kick.

TABLE 1

Percentage composition of diets

INGREDIENT	361	362	363	361-M	R-14	R-20	R-14-E
Casein, extracted [1]	6	———			0	———	
Peanut meal, extracted [1]	30				0		
Soybean meal, extracted [1]	0				40		
Sucrose	23.9		Same		31.9	Same	
Vitamin premix [2,3]	5		as		5	as	
Salts 5	5		diet		5	diet	
Lard	19		361		6	R-14	
Cod liver oil	1				2		
Ruffex	10				10		
L-Cystine	0.1	———			0		
DL-Methionine	0	0	0	0.4	0		
Choline chloride	0	0.04	0.12	0	0.12	———	
Acetylcholine Cl	0	0	0	0	0	1	0
α-Tocopheryl acetate	(10 mg daily)				0	0	0.01

[1] Exhaustive, continuous methanol extraction.

[2] The Vitamin premix contained pure vitamins added to sucrose to give the following levels per gram of diet: thiamine, riboflavin and pyridoxine, 3 μg each; Ca pantothenate, 17 μg; *i*-inositol, 200 μg; niacin, 30 μg; methyl-1,4-naphthoquinone, 0.3 μg. For diets 362-F, 363-F, and 361-M-F, 40 μg vitamin B_{12} and 2 mg folacin were added per kilogram of the corresponding diet without the letter "F."

[3] The vitamins were furnished by Merck and Company and A. E. Staley Manufacturing Company.

All of the choline-deficient rabbits developed a progressive muscular dystrophy between the 70th and 100th day on the diet. A typical creatine excretion curve and weight record are shown in figure 1. Creatine excretion gradually increased up to the 122nd day, at which time choline was added to the diet. Following the choline addition the creatine excretion dropped immediately and before the onset of growth. The

creatine output dropped below normal (to zero), fluctuated slightly, and thereafter held at a normal value. Data on the creatine and creatinine excretion of other rabits are given in table 2. In all cases creatine excretion gradually increased to levels many times greater than normal on the average, although there was considerable variation in individual values.

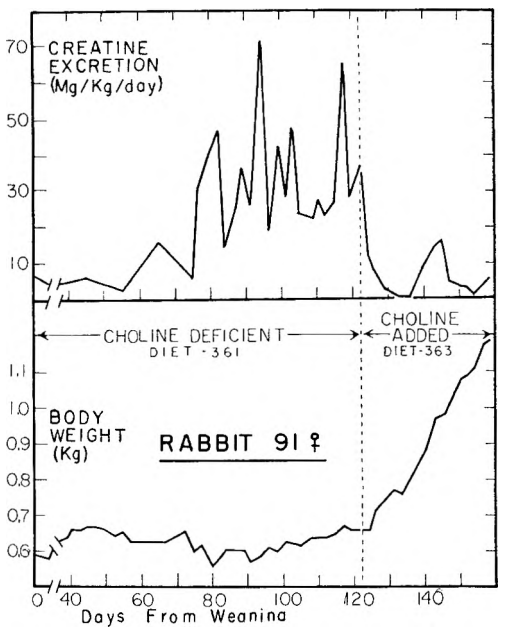

Fig. 1 Progressive development of high urinary creatine excretion due to prolonged choline deficiency. Note the immediate drop in creatine excretion when choline was added to the diet. At termination (158 days) the liver was still grossly cirrhotic and had a fat content of 42.7% on the dry basis.

Of special importance was the observation that creatinine excretion declined gradually but definitely as the animals progressed to a greater degree of dystrophy.

The legs of the choline-dystrophic rabbits became plastic; when placed in unnatural positions they remained for long periods. This is illustrated by the two poses in figures 3 and 4, which were typical of all of the animals. However, none lost complete control of their muscles, though movement was

at a minimum. The gross symptoms of the dystrophy due to choline deficiency were identical in all respects to those of the grade 2 dystrophy of the vitamin E deficiency (on a zero to 4 grade scale). When choline was added to the diet

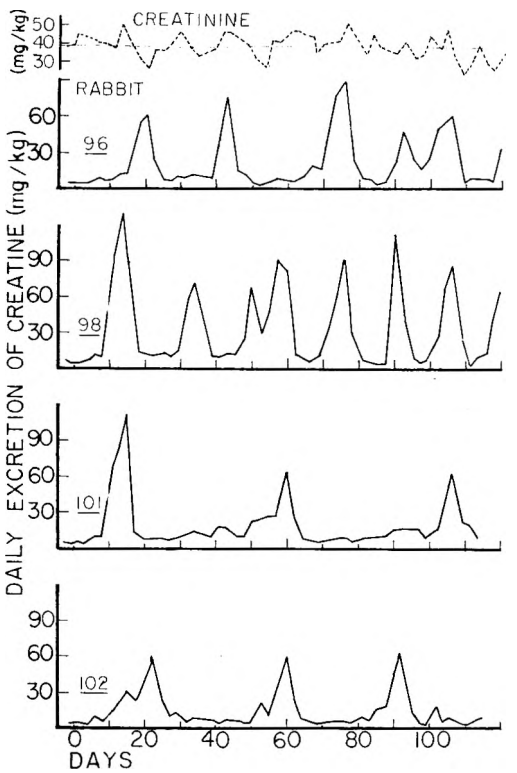

Fig. 2 Effect of dietary acetylcholine on the duration of cure of vitamin E deficiency. At each peak in the creatine excretion the rabbits were fed single doses of 30 mg DL-tocopherol acetate. Rabbits 96 and 98 were on diet R-14, while 101 and 102 were on diet R-20 (1% acetylcholine chloride). The dashed curve of creatinine excretion of rabbit 96 is typical. Body weights and average creatinine excretions are given in table 2.

of rabbit 91 (fig. 1) all signs of muscle weakness disappeared in 4 days. The legs could no longer be molded into bizarre positions, but instead had the feel of tightly coiled springs characteristic of normal rabbits.

TABLE 2

Creatine and creatinine excretion by choline-deficient rabbits

RABBIT NO.	DIET	BODY WEIGHT		TIME PERIOD		AVERAGE DAILY EXCRETION	
		Start	End	Days	Number of analyses	Creatinine	Creatine
		kg	*kg*			*mg/kg*	*mg/kg*
81	361	0.62	0.73	50	5	39.6	11.2
		0.73	0.91	50	21	33.7	19.5
		0.91	1.01	40	18	32.6	32.6
		1.01	0.93	41	18	31.8	38.8
84	361	0.66	0.99	50	5	45.6	7.8
		0.99	0.96	50	20	43.1	18.1
		0.96	0.95	40	18	38.6	44.2
		0.95	0.96	91	40	28.5	31.6
			Terminal:	1	1	12.0	67.8
85	361	0.53	0.79	50	5	43.6	12.2
		0.79	0.98	50	21	44.7	17.0
		0.98	1.00	20	8	42.0	27.7
		1.00	1.08	41	18	32.1	30.2
86	361	0.71	0.87	100	6	42.2	11.7
		0.87	0.89	30	12	38.4	15.0
		0.89	1.09	30	12	30.4	21.3
		1.09	1.17	33	14	27.3	33.9
91	361	0.58	0.60	75	4	37.3	8.8
		0.60	0.66	47	20	27.8	32.5
79	363	1.06	2.94	185	58	45.4	15.0
82	363	0.77	3.10	205	60	47.4	12.8
83	363	0.59	2.32	205	42	49.5	14.3
89	362	1.63	1.61	22	10	36.0	11.3
	363	1.61	1.87	28	11	42.9	11.5
90	362-F	1.61	1.59	22	8	48.4	13.9
	363-F	1.59	2.04	28	11	42.3	11.4
87	361-M	1.30	1.36	22	10	42.1	7.5
88	361-FM	1.53	1.56	22	10	41.2	12.3
96	R-14	0.67	2.69	125	53	37.9	....
98	R-14	0.78	2.49	125	53	38.8	....
100	R-14-E	0.44	2.20	125	42	38.6	8.5
101	R-20	0.61	2.73	125	53	39.3	....
102	R-20	0.55	2.35	125	53	39.8	....

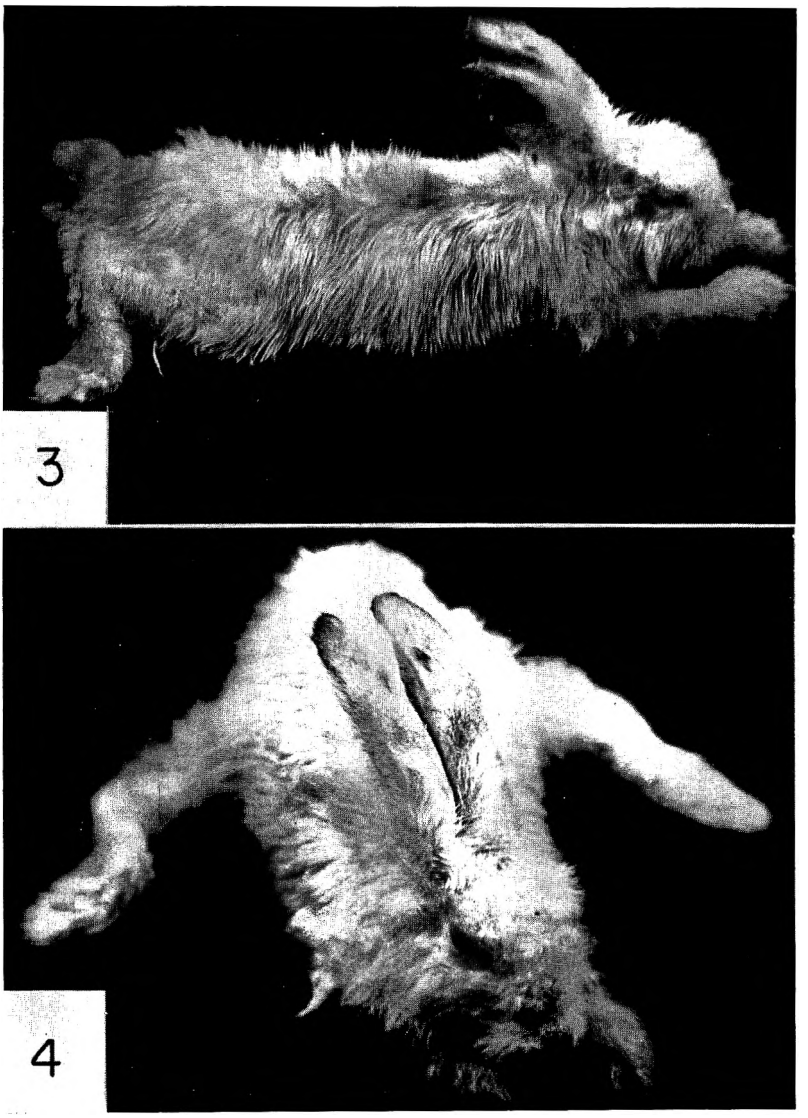

Fig. 3 Photograph of rabbit suffering from prolonged choline deficiency. Notice the plastic hind legs. Rabbit 84 after 200 days on the deficient diet. Side view.

Fig. 4 Photograph of the same rabbit as shown in figure 3 in a different pose to illustrate dystrophic condition.

The leg muscle was paler than normal in gross appearance. Microscopic studies of slides prepared from the muscle revealed considerable damage described as diffuse hyaline degeneration, which was essentially similar to that occurring in vitamin E dystrophy. There was loss of muscle fibers and an increase in fibrous connective tissue (fig. 5). The earliest change seemed to be a swelling of all or part of an individual muscle fiber followed by loss of striation (fig. 6). All or parts of the muscle fibers had a waxy appearance typical of hyaline degeneration.

Exclusion of vitamin E deficiency as a cause

It is well known that extensive damage to the liver, such as occurs in choline deficiency, interferes with the absorption of fat-soluble vitamins from the intestines. Conceivably, the creatinurea and dystrophy in the chronic choline-deficient rabbits resulted from a secondarily induced vitamin E deficiency. This possibility was minimized by feeding 10 mg DL,α-tocopheryl acetate daily to all rabbits, although the normal requirement is certainly not more than 1 mg daily (Hove and Harris, '47). Proof of absorption was obtained by analysis of plasma and tissues for vitamin E.

However, it is a fact that cirrhosis of the liver in both rats and rabbits does interfere with vitamin E absorption. Data showing that this occurs to a disturbing extent in rats with cirrhosis are presented in table 3. Severely cirrhotic rats,[2] on the choline deficient diets for as long as 30 weeks, had only 0.18 mg tocopherol per 100 ml of plasma. This is the level of severe vitamin E deficiency, although the diet was abundant in this factor. This finding has interesting implications as to the pathology of chronic choline-deficient rats. In preliminary work with rabbits, it had been found that after two to three months on the choline-deficient diet the vitamin E requirement increased greatly. Single doses of 30 mg α-tocopheryl acetate/kilogram body weight, which filled

[2] These rats were made available by Dr. M. J. Burns.

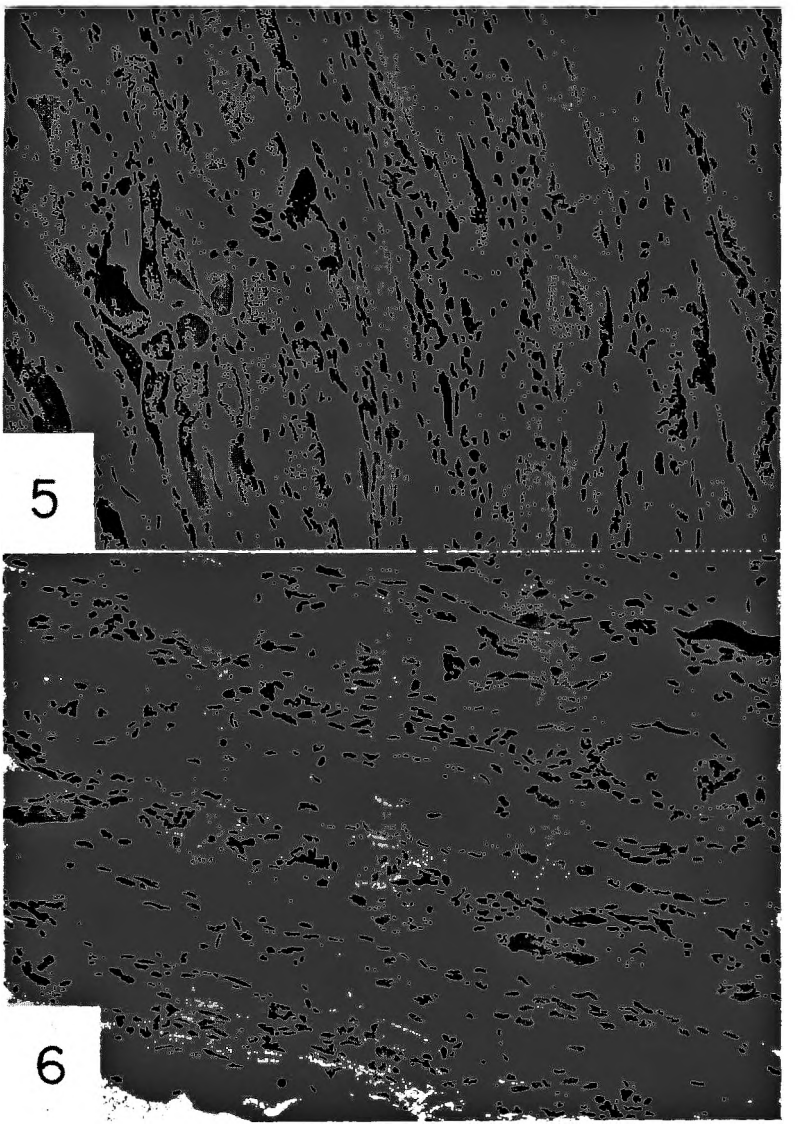

Fig. 5 Photomicrograph of a section of muscle from a chronic choline-deficient rabbit. There is loss of muscle fibers and an increase in fibrous connective tissue as well as hyaline degeneration. Hematoxylin and eosin. × 200.

Fig. 6 Photomicrograph of a section of muscle from a chronic choline-deficient rabbit to illustrate swollen and hyalinized fibers. Hematoxylin and eosin. × 200.

the vitamin E requirement of the added-choline controls for about 50 days, protected the choline-deficient rabbits against creatinurea for only about 10 days, and then only incompletely. Such rabbits had a flat "tolerance" curve following

TABLE 3

Plasma tocopherol of rats with liver cirrhosis

DIET	α-TOCOPHEROL IN DIET	LIVER CONDITION	WEEKS ON DIET	NO. OF RATS	PLASMA TOCOPHEROL
	%				*mg/100 ml*
Low choline [1]	0.01	Nodular cirrhosis	20	3	0.30
Low choline [1]	0.01	Nodular cirrhosis	30	4	0.18
Same plus 30 μg % vitamin B_{12}	0.01	Fatty, not cirrhotic	20	2	0.64
Same plus 30 μg % vitamin B_{12}	0.01	Fatty, not cirrhotic	30	2	0.73
Stock diet	0.006	Normal	30	2	1.05

[1] Same as diet 361, but without ruffex. The data in this table were obtained on rats supplied from the experiments of Dr. M. J. Burns. Appreciation is expressed to him for making these animals available

TABLE 4

The flat tocopherol "tolerance" curve in choline-deficient rabbits
(Averages of 2 animals per group after 90 days on diet)

DIET	AVERAGE BODY WEIGHT	PLASMA TOCOPHEROLS AT DAYS AFTER FEEDING A SINGLE DOSE OF 30 MG DL. α-TOCOPHEROL		
		0 day	2 days	6 days
	kg	*mg %*	*mg %*	*mg %*
361 (choline-low)	0.930	0.51	0.72	0.72
363 (0.12% choline)	2.27	0.80	2.18	1.29

single doses of α-tocopheryl acetate (table 4). When the tocopherol supplement was increased to 10 mg daily the creatinurea due to vitamin E deficiency was prevented.

The high (10 mg daily) dosage of tocopherol acetate was fed to the choline-deficient animals described in table 2, and

this resulted in a normal plasma tocopherol level, even though this level was only about half of that observed in the added-choline controls receiving the same absolute level of tocopherol. The tissues of the choline-deficient rabbits were also rich in tocopherol (table 5). These results would seem to exclude a secondarily induced vitamin E deficiency as a cause of the muscular dystrophy noted in the choline-deficient rabbits.

TABLE 5

Plasma and tissue tocopherol of choline-deficient rabbits with dystrophy as compared with vitamin E-deficient and normal rabbits

DIET	α-TOCOPHEROL FED DAILY	TOCOPHEROL CONTENT OF				
		Blood plasma	Liver fat	Brain fat	Kidney fat	Muscle fat
	mg	*mg %*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*
361 (low choline) for 162 days (av. of 3 animals)	10	1.13				
363 (plus choline) for 162 days (av. of 3 animals)	10	2.08				
361 for 231 days	10	1.83	3.40 (43.6)[1]	0.69	1.60	2.69 (3.0)[1]
R-14 for 42 days (av. of 3 animals)	0	0.16	0.24 (10.7)[1]	0.06	0.05	0.45 (16.5)[1]
R-14-E for 67 days (av. of 2 animals)	1	0.69	0.41 (12.9)[1]	0.18	0.76	0.57 (6.1)[1]

[1] Total lipid of tissue as per cent, dry basis.

The influence of acetylcholine on the vitamin E requirement of rabbits

If the dystrophy of vitamin E deficiency is related to inadequate acetylcholine formation, as postulated, a preformed supply of acetylcholine ought to prevent or lessen the vitamin E-deficiency dystropy. Rabbits were placed on a vitamin E-deficient diet, R-14, and allowed to develop recurring states of the deficiency as measured by creatine excretion. Each time that the creatine excretion had risen to more than 60

mg/kg/day, a single dose of 30 mg of DL,α-tocopheryl acetate in olive oil was given orally. Acetylcholine chloride had been added to the diet of some of the rabbits at levels of 0.1% and 1.0%, even though it did not seem plausible to expect acetylcholine added to the diet to be absorbed as such, and to penetrate to the necessary sites of action in the body. However, this treatment did result in a marked delay in the reoccurrences of creatinurea after the single doses of tocopherol acetate (fig. 2). The data on the lower level of dietary acetylcholine are not shown, but were intermediate in effect. The creatinine excretion in vitamin E deficiency was not altered from normal. A chart of the creatinine excretion of a single, very typical rabbit is included in figure 2, while the average values of this and other animals in this series are given in table 2.

DISCUSSION

The fact that chronic choline deficiency in rabbits produced a progressive muscular dystrophy supports the premise that diminished acetylcholine is the key to the pathogenesis of the dystrophies. This study is perhaps the first to show a nutritional muscular dystrophy in rabbits other than that produced by a simple vitamin E deficiency, and care has been taken to exclude a vitamin E deficiency as a possible cause or contributing factor.

In some respects the choline dystrophy is more closely related to human progressive muscular dystrophy than is the vitamin E dystrophy. The creatine excretion developed slowly and increased progressively; but perhaps of greater significance was the concomitant gradual decrease in creatinine excretion. This effect is also very characteristic of the human dystrophies (Hoagland et al., '45), while it does not occur in the vitamin E dystrophy in rabbits. Although the choline dystrophy is similar in certain points to the human dystrophies, the latter certainly cannot be the result of simple choline deficiency, since in human subjects growth is essentially normal and liver pathology is not characteristic.

The significance of the present findings is to show that muscular dystrophy may result from dietary deficiencies other than a vitamin E deficiency.

The choline dystrophy did not begin to develop until the animals had been on the diet for more than 70 days. The choline requirement for acetylcholine production is undoubtedly very small in comparison with that for phospholipid formation and other functions, and it has therefore been assumed that the delay in muscular dystrophy development reflected the need for intensification of the deficiency. However, another interpretation is possible. Cirrhosis of the liver developed about the same time as the dystrophy, and impaired liver function may have been causal to the dystrophy. It was pointed out (Hove, '52b) that the synthesis of creatine was *decreased* in liver slices from vitamin E-deficient rats, *in vitro*. If inability to form "active" creatine in the liver triggers the development of muscular dystrophy, then cirrhosis, by lessening the amount of active liver tissue, may bring about the same result. A concept similar to this was advanced by Aloisi and Bonetti ('52) who noted muscular lesions in chronic choline-deficient rats. However, the interpretation of their data is complicated by the observation in the present paper that such rats are suffering from a severe vitamin E deficiency, as judged by plasma tocopherol levels (table 3) and in spite of an adequate dietary level of vitamin E.

The rabbits reported in the present paper did not have a vitamin E deficiency. Whether or not the muscular dystrophy noted in these animals was a primary result of the choline deficiency, or an indirect effect of the liver cirrhosis with its concomittant decrease in normal liver tissue, must be answered by further work. However, the addition of choline to the diet of the choline-dystrophic rabbit resulted in complete clinical cures of the dystrophy within two or three days. It does not seem possible that the extensive damage to the liver could have been repaired in so short a time.

SUMMARY

Progressive muscular dystrophy developed in rabbits that had been fed a choline-deficient diet for more than 70 days. Creatine excretion gradually increased to more than 40 mg/kg/day, while the creatinine excretion decreased to less than two-thirds of the normal value. The posterior extremities became flaccid and extremely weak, although complete loss of muscle control did not occur. Histologic studies on the muscle have shown hyaline degeneration and other lesions characteristic of muscular dystrophy. The addition of choline to the diet brought about a rapid cure of the clinical dystrophy and creatinurea within three days. Vitamin E deficiency as a contributing cause of this dystrophy was ruled out by feeding 10 times the normal amount, and determining the tocopherol content of plasma, muscles and other organs.

For comparison, data on the dystrophy due to a vitamin E deficiency are given, and it has been shown that the addition of 1% acetylcholine to the diet lessened the vitamin E requirement of rabbits.

ACKNOWLEDGMENT

Valuable technical assistance was given by Jack McElyea.

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FACTORS INFLUENCING THE GROWTH OF CHICKS AND POULTS FED RATIONS CONTAINING RAPESEED OIL MEAL [1]

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TWO FIGURES

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Rapeseed oil meal has been shown to be a satisfactory substitute for meat meal in chick rations in amounts up to 14% of the total ration, although 20% was unsatisfactory (Pettit et al., '44). Five per cent of rapeseed oil meal was satisfactory for chicks in experiments by Kondra and Hodgson ('48). Turner ('46, '48) showed that rapeseed oil meal contained a goitrogen active for the chick and that the meal depressed growth when fed at levels from 10 to 40% of the ration. He interpreted the data to indicate that the depressed growth resulted from a lack of thyroxine secretion.

Blakely and Anderson ('48a) fed rapeseed oil meal to turkey poults at 4, 10 and 20% of the ration and observed thyroid enlargement at all of the levels fed. Protamone reduced the size of the thyroid gland in poults fed 20% rapeseed oil meal and "induced growth more nearly normal than rapeseed oil meal without the addition of protamone."

An anti-thyroid compound has been isolated from rapeseed and has been identified as L-5-vinyl-2-thio oxazolidane (Astwood et al., '49; Carroll, '49).

Matet et al. ('49) reported that rapeseed meal was toxic to albino rats and that the toxic glucosides could be removed

[1] A preliminary report was presented at the meeting of the American Institute of Nutrition in Chicago, April 9, 1953.

by extraction with 70% ethanol or by fermentation and subsequent solvent extraction.

Since work on linseed oil meal in this laboratory has indicated the presence of a growth inhibiting factor (Kratzer and Williams, '48) it seemed desirable to determine whether the growth inhibiting factor in rapeseed oil meal is similar to that in linseed oil meal and if it is not, to determine the factors limiting the use of rapeseed oil meal in rations for chicks.

EXPERIMENTAL

Two commercially processed rapeseed oil meal samples were used and contained 37.4 and 43.8% crude protein respectively. S.C. White Leghorn cockerel chicks were used in most experiments while New Hampshire chicks and Bronze poults were each used in one experiment. The birds were kept in electrically heated batteries with raised wire floors and were supplied feed and water ad libitum. The thyroid glands were removed immediately after the birds were killed, in experiments in which the weights were determined. The glands were trimmed, placed in Bouin's solution for a few days and then carefully blotted dry and weighed.

Effect of rapeseed oil meal upon the growth of chicks

In order to establish the fact that the rapeseed oil meal which we had available would depress growth, it was tested by substituting it for soybean oil meal in a practical type chick ration. Experiments 1, 2 and 3 were continued for 40, 22 and 14 days respectively. As the amount of rapeseed oil meal in the ration was increased, growth of the chicks decreased until approximately 75% of normal growth was obtained when 35% of rapeseed oil meal was used (fig. 1).

Effect of pyridoxine and water treatment

Since either pyridoxine supplementation or treatment with water (Kratzer and Williams, '48) will effectively counteract the growth inhibitor in linseed oil meal, similar procedures

were used with rapeseed oil meal. Neither supplementation with pyridoxine nor water treatment caused any improvement in the growth of chicks fed either 22 or 30% of rapeseed oil meal (table 1).

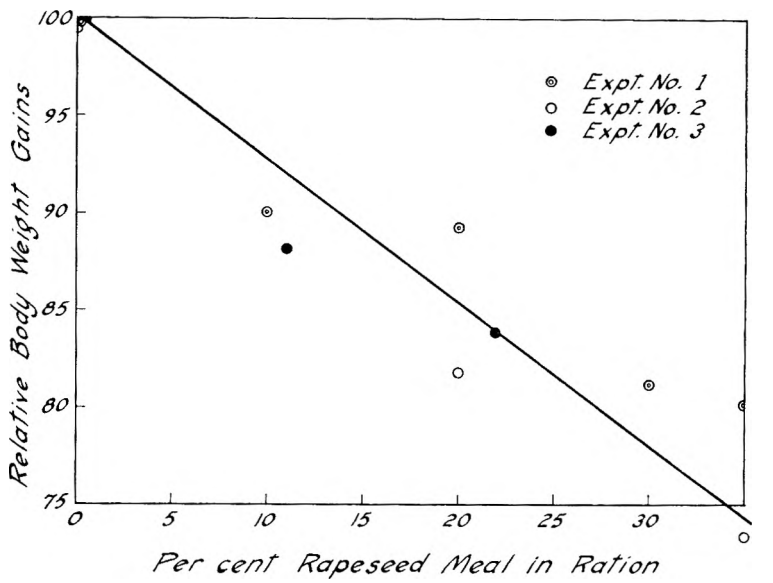

Fig. 1 The effect of level of rapeseed oil meal in the ration upon body weight gains of chicks.

TABLE 1

The effect of water-treatment and pyridoxine supplementation of rapeseed oil meal upon the growth of chicks

SUPPLEMENT	EXPT. 4		EXPT. 5	
	Level	Gain	Level	Gain
	%	*gm*	*gm*	%
Rapeseed oil meal	30	122	22	136
Rapeseed oil meal Pyridoxine	30 0.00375	121	22 .00375	132
Water-treated rapeseed oil meal	30	124	22	135
Duration, days		11		14
Number of chicks per group		6		10

Effect of protamone upon growth and thyroid weight of chicks fed rapeseed oil meal

Turner ('48) found that the thyroids of chicks were progressively larger as the intake of rapeseed oil meal was increased, and that the growth of the chicks was decreased. To study this relationship further, protamone was added at

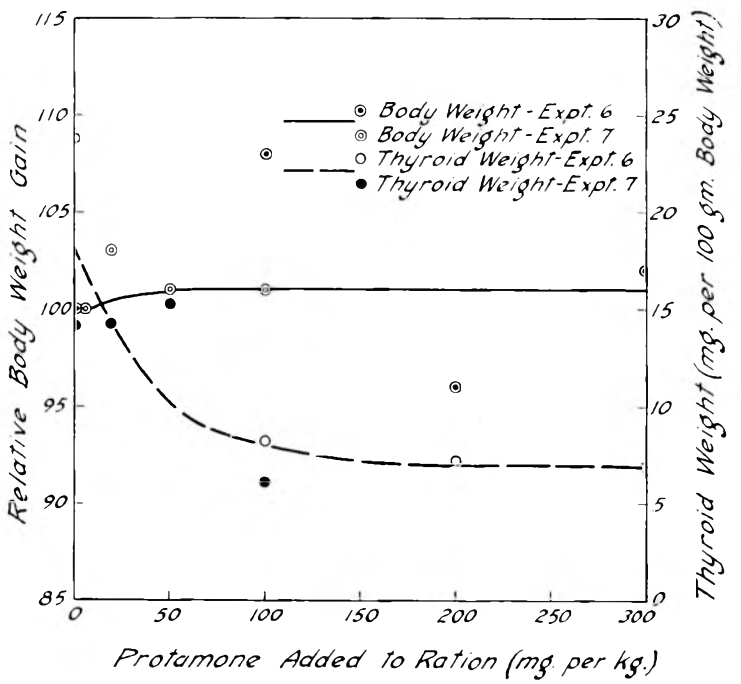

Fig. 2 The effect of protamone in the diet upon body weight gains and thyroid weights of chicks fed ration containing rapeseed oil meal.

various levels to a practical type ration containing 30% of rapeseed oil meal. The thyroid weight per 100 gm of body weight was reduced markedly by 100 mg of protamone per kilogram and there was little additional effect at 200 and 300 mg levels (fig. 2). The body weight gains of the birds were rather variable in experiment 6 but were quite consistent in experiment 7, showing only a very slight growth increase when protamone was added to the ration.

Thyroid weights of birds fed the basal ration to which 50 mg of potassium iodide were added were 17.7 and 16.6 mg per 100 gm of body weight in experiments 6 and 7 respectively. Iodine is thus unable to overcome the goitrogen in rapeseed oil meal while 100 mg of protamone per kilogram reduced the thyroid size to normal or below.

TABLE 2

Effect upon the growth of chicks of the addition of amino acids to a ration containing rapeseed oil meal as the only source of protein

SUPPLEMENT	LEVEL	PERCENTAGE DAILY GAIN				
		Expt. 8	Expt. 9	Expt. 10	Expt. 11	Expt. 12
	%					
None		5.4	6.5	5.6	4.7	5.9
Mixture A						
DL-Lysine-HCl	0.45					
L-Arginine	0.2					
DL-Methionine	0.2	6.8	6.4	6.1	5.2	6.0
L-Cystine	0.2					
DL-Tryptophan	0.1					
Mixture A without lysine-HCl		7.0				
Mixture A without arginine		5.4				
Mixture A without methionine		6.9				
Mixture A without cystine		7.2				
Mixture A without tryptophan		6.9				
L-Arginine	0.2		6.4	5.4		
DL-Lysine-HCl	0.45				4.9	
L-Arginine	0.2					
Methionine	0.2					
Cystine	0.2				4.2	
Tryptophan	0.1					
Stock mash		6.2	7.4	7.9	6.5	7.5
Duration, days		10	12	10	15	10
Number of chicks per group		7	7	7	7	7

Amino acid supplementation of rapeseed oil meal

Five experiments were conducted in which rapeseed oil meal contributed the only protein in a purified ration (Kratzer et al., '47) and various amino acids were added to this basal ration. In experiment 8 (table 2) there was a slight improve-

ment in growth when a mixture of lysine, arginine, methionine, cystine and tryptophan was added. The chicks in the basal group were reluctant to eat the experimental ration at first, which caused poor gains in this group. Their growth later in the experimental period was much better. There was no marked depression in growth when any single amino acid was omitted from the mixture. In other trials there was no significant response to the addition of an amino acid mixture to the basal diet containing rapeseed oil meal. It was thus concluded that rapeseed oil meal protein was itself an adequate amino acid source for the growth of chicks.

Amino acid supplements for practical rations containing rapeseed oil meal

Experiments in which it was established that rapeseed oil meal depresses growth were conducted with rations containing practical feedstuffs (Turner, '48; Blakely and Anderson, '48; Pettit et al., '44). Experiments at this laboratory indicated that fish meal was effective in supplementing rapeseed oil meal to improve growth. Since this effect suggested that an amino acid deficiency was being corrected, two experiments were conducted with chicks to determine whether lysine or methionine or both would supplement rapeseed oil meal in a practical type ration. The ration for chicks was composed of the following: rapeseed oil mean 26.0; ground barley 10.0; ground wheat 10.0; wheat bran 5.0; alfalfa meal 2.5; fish meal, 2.5; ground limestone, 2.25; dried whey, 2.0; steamed bone meal, 2.0; salt 0.5; choline chlordie (25%), 0.25; fish oil (2250 A, 300 D), 0.15; butyl fermentation product 0.025; manganese sulfate, 0.025; crude aureomycin, .002 gm and ground corn to equal 100 gm after the supplements were added.

Methionine, lysine and protamone additions were made to the basal diet as shown in table 3, and there were 10 chicks per group in each of the two trials. S.C. White Leghorn cockerels were used in one experiment and New Hampshire chicks of mixed sexes in the other.

In the trials with S.C. White Leghorns (table 3) lysine gave a highly significant growth response with the single exception of its addition to the chick ration containing protamone. Lysine gave a highly significant growth increase in the trial with New Hampshire chicks except in the presence of methionine alone. Methionine did not produce a significant growth response in either trial. Protamone produced a highly

TABLE 3

The effect of 0.2% L-lysine, 0.1% DL-methionine and 0.01% protamone added singly and in combinations to a ration containing rapeseed meal upon growth and thyroid weight in chicks

SUPPLEMENT	S. C. WHITE LEGHORN		NEW HAMPSHIRE	
	Daily gain	Thyroid weight	Daily gain	Thyroid weight
	%	*mg/100 gm body wt.*	%	*mg/100 gm body wt.*
None	5.51	7.3	5.32	4.3
Lysine	5.85	5.5	5.81	4.8
Methionine	5.59	3.2	5.56	5.6
Lysine + methionine	5.99	4.1	5.75	5.6
Protamone	5.86	0.6	5.37	1.1
Protamone + lysine	5.98	0.6	5.79	1.7
Protamone + methionine	5.72	0.9	5.51	2.5
Protamone + lysine + methionine	6.01	0.5	5.92	1.3
Stock mash control	6.14	2.3	5.89	2.5
Duration, days	21		26	
Number of chicks per group	10		10	
Least difference for significance 5% level	0.19		0.20	
1% level	0.26		0.27	

significant growth response when added to the unsupplemented chick ration in the Leghorn experiment. In all other instances no response was noted. The thyroid weight was reduced by the addition of protamone as was expected from earlier work. Methionine caused a slight reduction in the thyroid weight when it was added to the chick rations containing no protamone in the Leghorn trial but had no effect in the experiment with New Hampshire chicks.

A single trial was conducted with poults in which lysine, methionine and protamone supplements were added to a practical type ration containing rapeseed oil meal. The ration was very similar to that used in the chick studies except that the amount of rapeseed oil meal was increased so that the ration provided about 25% protein. The results (table 4) were very similar to those obtained with chicks. Lysine, but not methionine, produced an increase in the rate of growth.

TABLE 4

The effect of 0.2% L-lysine, 0.1% DL-methionine and 0.01% protamone, added singly and in combinations, upon growth, pigmentation and thyroid weight of poults. (12 poults per group for 22 days)

SUPPLEMENT	DAILY GAIN	WHITE BAR SCORE [1]	THYROID WEIGHT
	%		*mg/100 gm body wt.*
None	6.26	0.67	6.9
Lysine	6.71	0.10	6.3
Methionine	6.35	0.83	10.5
Lysine + methionine	6.90	0.25	6.7
Protamone	6.23	0.81	0.9
Protamone + lysine	6.76	0.15	0.8
Protamone + methionine	6.27	0.56	1.2
Protamone + lysine + methionine	6.88	0.23	0.6
Poult starter	7.25	0.00	1.7
Least difference for significance at 5% level	0.23		
1% level	0.32		

[1] Maximum severity of lack of pigmentation in remiges equals score of 1.0.

Protamone gave no response. The severity of white bar, a symptom of lysine deficiency (Kratzer et al., '50) was markedly reduced by lysine supplementation. The thyroid weight was reduced by protamone but was not influenced by the amino acid supplements.

Analyses of rapeseed oil meal have given 1.39% methionine by the method of McCarthy and Sullivan ('41), as modified by Grau and Almquist ('45) and 1.83% lysine by the method of (Horn et al., '47) employing *Leuconostoc mesenteroides* P-60.

DISCUSSION

The effect of rapeseed oil meal in reducing the growth rate of chicks and poults as reported previously by Blakely and Anderson ('48a, '48b), Pettit et al. ('44), and Turner ('48) has been confirmed by using it as a replacement for soybean oil meal in a practical type ration.

The presence of a goitrogen in rapeseed oil meal has been reported by Turner ('46) and Blakely and Anderson ('48a); Astwood et al. ('49) and Carroll ('49) were successful in isolating and identifying the goitrogen. If the goitrogen is responsible for the poor growth-promoting activity of the meal as implied by Turner ('48) and Blakely and Anderson ('48b), growth should be markedly improved by the addition of a thyroactive material such as protamone. Our results showed that protamone was highly effective in reducing the weight of the thyroid gland but had very little effect upon the rate of growth of chicks and poults.

Matet et al. ('49) reported that a glucoside toxic to rats could be removed from linseed oil meal by extraction with 70% alcohol. The effect of this material upon chicks and poults was not investigated; however, it was shown that rapeseed oil meal does not contain the growth inhibitor which has been noted in linseed oil meal. There was no improvement in the growth of chicks which were fed rapeseed oil meal treated with water or supplemented with pyridoxine. Both of these procedures are effective in counteracting the growth inhibitor in linseed oil meal (Kratzer and Williams, '48).

Rapeseed oil meal was a adequate source of amino acids for the chick where it was the sole source of protein. However, in practical rations containing many feedstuffs which are deficient in lysine and methionine, lysine was definitely a growth-limiting factor. Significant growth responses were noted in both chicks and poults. Using 1.83% as the lysine content of rapeseed oil meal and average values for other feedstuffs as a basic for calculation, the chick rations contained 0.76% lysine. This indicates a deficiency of lysine when we compare it with the requirements of 0.9% as reported by

Almquist ('48). The basal ration was calculated to contain 0.62% methionine, which is adequate in reference to the requirement of 0.5%. Similarly, the poult basal ration contained 1.14% lysine and 0.83% methionine compared with requirements of 1.3% and 0.5%. The addition of 0.2% L-lysine markedly improved growth and reduced the severity of the white bar. It was probably not completely adequate as shown by the fact that the white bar was not entirely prevented.

The possibility exists that the samples of rapeseed oil meal which were used did not contain as much of the goitrogen as samples used by other investigators since the thyroid enlargement which resulted was not as extreme as that reported by Turner ('46, '48). Even if this difference exists, however, our results show that rapeseed oil meal is marginal as a source of lysine and does not contain a sufficient excess to be used in the usual practical chick ration without causing a deficiency of this amino acid.

Potassium iodide was ineffective in decreasing the thyroid weight of chicks fed rapeseed oil meal. This is in contrast to the ability of potassium iodode to counteract the goitrogen in soybeans (Wilgus et al., '41).

SUMMARY

Rapeseed oil meal depressed the growth of chicks approximately 25% when substituted for 35% soybean oil meal in the ration. Growth was not improved by water treating the rapeseed oil meal or by supplementing it with pyridoxine.

Rapeseed oil meal caused an enlargement of the thyroid gland, which was not counteracted by feeding potassium iodide. Protamone added to the ration containing rapeseed oil meal resulted in a thyroid of normal weight but had little or no effect on the growth of the chicks.

Rapeseed oil meal was an adequate source of amino acids for chicks when used as the only source of protein. Practical rations containing rapeseed oil meal needed additional lysine for optimum growth of chicks and for optimum growth and feather pigmentation in poults.

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VITAMIN B_{12} CONTENT OF ORGAN MEATS [1]

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INTRODUCTION

Improvements in the method of liberating and assaying vitamin B_{12} microbiologically have been reported previously (Scheid and Schweigert, '51; Scheid, Andrews, and Schweigert, '52) for beef liver and kidney, and pork, beef, and lamb muscle cuts. These studies have been continued, and the effect of KCN, $NaNO_2$, thioglycollic acid or ascorbic acid additions to the sample prior to assay or to the assay medium prior to sterilization has been investigated with *Lactobacillus leichmannii* 327 as the test organism. The method adopted was subsequently applied to the assay of the vitamin B_{12} in composite samples of organs from each of two sources for beef and pork, and from one source for lamb.

The samples were also assayed for residual vitamin B_{12} potency after alkali treatment. Pancreas is shown to contain a considerable amount of vitamin B_{12} activity after alkali treatment when measured with *L. leichmannii* but not with *Euglena gracilis* as the test organism.

EXPERIMENTAL

The method of selection of the organ meat samples, their preparation, and proximate analyses have been described earlier (Scheid et al., '53). The organs studied included liver, heart, kidney, brain, spleen, pancreas, and lung of beef, lamb,

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and pork, and liver from old cows and sows. The samples represent a finely ground composite of the entire organ from each of 10 animals for each species or a one-pound portion of liver from each of 10 animals.

The basal medium used in the microbiological assay for vitamin B_{12} was the amino acid medium containing thioglycollic acid described previously (Scheid and Schweigert, '51). Crystalline vitamin B_{12} was used as the standard, and crystalline vitamin B_{12}b and liver extract were used as secondary standards. The tubes were sterilized in the autoclave for 10 minutes at 15 pounds pressure, cooled, and inoculated with a drop of a washed culture of *L. leichmannii* 327.

In the initial experiments a number of supplements reported to stabilize the vitamin B_{12} potency of samples (by conversion of vitamin B_{12b} —— hydroxy-cobalamin —— into a more stable form) were tested. The effect of cyanide upon the microbiological assay of vitamin B_{12} has been shown by Veer et al. ('50), Wijmenga et al. ('50), Lens et al. ('52), Loy and Kline ('51), Loy et al. ('52), and Cooperman et al. ('51) to be due presumably to the conversion of the vitamin derivatives in the sample to vitamin B_{12} (cyano-cobalamin). Thioglycollic acid, a reducing agent, has also been used to protect vitamin B_{12}. It appeared likely that thioglycollic acid added to the sample prior to homogenization would offer a protective action and thereby stabilize the vitamin throughout the assay. In our experiments 20 μg of KCN or 20 mg of thioglycollic acid were added to the homogenized sample (2 gm of sample in 70–80 ml of 0.1M phosphate buffer at pH 7.0) before autoclaving. Equivalent amounts of KCN or thioglycollate, based on dilution of the sample, were added to the standard before assay. The results were compared with those of tryptic digestion or of autoclaving the samples in phosphate buffer. The autoclaving period used was 5 minutes. Comparative studies were also made with the samples autoclaved at pH 7 and 4.5 with 5- and 30-minute autoclaving periods. $NaNO_2$ was also tested according to the method of Prier et al. ('52) and compared with the other methods.

Subsequent to these studies, the vitamin B_{12} potency of the organ meats was determined before and after alkali treatment. Alkali treatment was used to destroy the vitamin B_{12} in the samples (Hoffmann et al., '49; Scheid and Schweigert, '50) and permit measurements of the residual vitamin B_{12} potency, presumably due to desoxyribosides. In view of the high activity observed for pancreas after alkali treatment, these samples were also assayed with *E. gracilis* which does not respond to desoxyribosides.

RESULTS AND DISCUSSION

None of the procedures tested (additions of thioglycollic acid or KCN to the sample and basal medium at pH 7 or 4.5 or tryptic digestion) or the method of Krieger ('52) resulted in an increase or decrease in the assay values as compared to those observed for samples prepared in phosphate buffer and autoclaved for 5 minutes prior to assay. Similarly, in a separate study (Worland and Schweigert, '52, unpublished data) no marked effects of ascorbate additions to the media or tryptic digestion of the samples were noted on the vitamin B_{12} potency of liver, kidney, and muscle measured with *L. leichmannii* or *E. gracilis*. Some increase, although quite variable, was observed when nitrite was added to the samples. Nitrite addition to the standard (in amounts equivalent to those provided by the samples) also gave variable responses. In view of these results and the difficulty of providing equivalent levels of nitrite to the standard when the samples varied in vitamin B_{12} potency (and thus in nitrite necessitated by different dilutions of the sample), the method did not merit adoption for assay purposes.

A 5-minute autoclaving period for the sample at pH 7.0 was therefore used, and the results are given in table 1. Results are also given for each sample assayed after alkali treatment. The vitamin B_{12} potencies of the samples from different sources were in good agreement, and beef and pork liver from animals of different ages were of compable B_{12} potency. Liver and kidney are the richest sources of vitamin

B_{12}. The vitamin B_{12} potency of beef organs was higher than that for pork. The vitamin B_{12} potency of spleen, heart, brain, and lung was slightly higher than that observed previously for beef or pork muscle tissues (Scheid and Schweigert, '51).

TABLE 1

Vitamin B_{12} potency of organ meats

(Values expressed as micrograms of vitamin B_{12} per 100 gm of undried sample)

SOURCE [1]	MATERIAL	B_{12} POTENCY [2]		SOURCE	MATERIAL	B_{12} POTENCY	
		Fresh	Alkali			Fresh	Alkali
1	Beef liver	60	0.2	1	Beef spleen	5.2	1.0
2	Beef liver	70	6.0	2	Beef spleen	5.0	2.0
1	Old cow liver	55	3.5	2	Sheep spleen	6.7	2.0
2	Old cow liver	57	5.0	1	Pork spleen	4.1	2.6
2	Sheep liver	35	1.9	2	Pork spleen	4.0	1.4
1	Pork liver	27	1.0				
2	Pork liver	19	2.0	1	Beef heart	10.6	0.4
1	Old sow liver	30	2.0	2	Beef heart	8.8	0.8
2	Old sow liver	20	4.0	2	Sheep heart	5.2	0.5
				1	Pork heart	3.0	0.5
1	Beef kidney	32	1.0	2	Pork heart	1.8	1.0
2	Beef kidney	24	1.0				
2	Sheep kidney	26	1.2	1	Beef brain	4.9	0.4
1	Pork kidney	7.1	0.7	2	Beef brain	4.5	0.5
2	Pork kidney	6.2	1.0	2	Sheep brain	7.3	0.8
				1	Pork brain	3.3	0.4
1	Beef pancreas	14	5.5	2	Pork brain	2.2	1.0
2	Beef pancreas	14	4.0	1	Beef lung	4.0	1.0
2	Sheep pancreas	19	10.0	2	Beef lung	2.7	2.0
1	Pork pancreas	13	5.7	2	Sheep lung	5.0	1.0
2	Pork pancreas	20	7.3	1	Pork lung	2.1	1.0
				2	Pork lung	2.1	1.0

[1] No. 1 and no. 2 refer to the two commercial sources from which the organs were procured.

[2] Four or more assays in all cases.

It will be noted that a considerable amount of the vitamin B_{12} potency of the samples was stable to alkali treatment for some of the samples, and in particular, for pancreas. Alkali treatment has been used previously to differentiate between vitamin B_{12} activity (alkali-labile) and desoxyriboside activity (alkali-stable) for *L. leichmannii*. In our experience, purified

preparations of vitamin B_{12} or liver extract consistently have no vitamin B_{12} activity after alkali treatment; however, samples such as organ meats or rat liver have considerable activity, which is variable from experiment to experiment after alkali treatment. Since larger amounts of the sample are required after alkali treatment, stimulatory materials other than vitamin B_{12} or desoxyribosides may be introduced. It is also possible that forms of vitamin B_{12} in these lower potency samples are not completely destroyed by the alkali treatment. Thus, alkali lability may give a fairly good indication of the true vitamin B_{12} potency of certain samples, particularly of higher vitamin B_{12} potency. However, alkali treatment does not appear justified at present as a technique for routine application in measuring the specific vitamin B_{12} potency of samples. This seems particularly true for lower potency materials in which considerable vitamin B_{12} activity remains after alkali treatment. For the present paper, therefore, the data obtained without alkali treatment have been used for comparative purposes. Further study and comparisons with other methods, such as isotopic dilution techniques with vitamin B_{12} labelled with radioactive cobalt, may afford sufficient evidence to evaluate the reliability of alkali treatment adequately.

Because of the high vitamin B_{12} activity of pancreas after alkali treatment, the vitamin B_{12} potency of the pancreas samples was determined with and without alkali treatment with the use of both *L. leichmannii* and *E. gracilis* as test organisms. In these studies (table 2), the vitamin B_{12} potency of the samples was lower with *E. gracilis* with both sample treatments. These results indicate that most, but not all, of the vitamin B_{12} potency of pancreas remaining after alkali treatment is due to desoxyribosides (active for *L. leichmannii* and inactive for *E. gracilis*). Other organ meats assayed after alkali treatment were not markedly lower in vitamin B_{12} potency by *E. gracilis* assay as compared to *L. leichmannii* in our experience (Scheid et al., '51; Worland, '52). The lack of agreement in the values obtained with *E. gracilis* in

the fresh samples and those obtained with *L. leichmannii* (by subtracting the value obtained after alkali treatment from the value obtained before alkali treatment) for pancreas (table 2) has also not been observed for other samples (Scheid et al., '51; Worland, '52). For example, in the latter study the vitamin B_{12} potency of beef liver and kidney was 41 and 17 μg per 100 gm with *L. leichmannii*, and 39 and 16 μg per 100 gm, respectively, with *E. gracilis* as the test organism, while the corresponding values with *L. leichmannii* after alkali treatment of the samples were 1.9 and 1.3, and with *E. gracilis*

TABLE 2

Vitamin B_{12} potency of pancreas before and after alkali treatment

(Expressed as micrograms per 100 gm)

MATERIAL	L. LEICHMANNII		E. GRACILIS	
	Fresh	Alkali	Fresh	Alkali
Beef pancreas	13.6	5.5	4.8	0.4
Beef pancreas	13.5	4.0	3.6	0.1
Sheep pancreas	18.5	10.0	6.2	0.8
Pork pancreas	13.2	5.7	3.3	0.6
Pork pancreas	19.6	7.3	..	0.2

1.6 and 0.7 μg per 100 gm, respectively. In these instances small amounts of alkali-stable material are present relative to the potency before alkali treatment. These results add further evidence for the need of subjecting methods of assay, as for vitamin B_{12}, to continuing critical scrutiny. It will be particularly important, for example, to determine the vitamin B_{12} potency of pancreas by microbiological as compared to animal assay.

Values for the vitamin B_{12} potency of meats have been published from several laboratories. These are presented in table 3. In view of the use of different samples and methods of assay, the values are in rather good agreement in most cases.

TABLE 3

Comparative B_{12} potencies of organ meats

(All values expressed as micrograms per 100 gm of fresh sample)

MATERIAL	LABORATORY								
	1	2	3	4	5	6	7	8	
Beef spleen	2.7							5.2	5.0
Beef brain	4.3							4.9	4.5
Beef heart	17.6			3.0				10.6	8.8
Beef liver	105	26, 20, 24 24, 25	15	14	152	47, 54, 99 62, 31, 41	45	60	70
Beef pancreas	12							14	14
Beef lung	1.8							4.0	2.7
Beef kidney	1.7		20	10		23, 15, 19, 20	19	32	24
Beef muscle	0.25		2.0, 3.0 3.0			2.3, 3.4, 1.8, 1.9, 1.3, 2.9	2.0		
Pork liver		2,3			15			27	19
Pork spleen		7.0						4.1	4.0
Veal brain		1.5							
Veal heart		8.0, 7.0							

1. Tastaldi, '50.
2. Tappan et al., '50.
3. Lewis et al., '49.
4. Thompson et al., '50.
5. Guttmann and Vandenheuvel, '52.
6. Scheid and Schweigert, '51; Scheid et al., '52.
7. Worland, '52.
8. Present studies.

SUMMARY

The microbiological method of assay for vitamin B_{12} using *L. leichmannii* 327 as the test organism has been further investigated. Additions of KCN, $NaNO_2$ or thioglycollic acid to the sample or assay medium did not improve the assay

method over that of short term autoclaving ad subsequent assay with the use of an amino acid medium containing thioglycollic acid.

The vitamin B_{12} potency of composite samples of liver, kidney, pancreas, spleen, heart and lung, from beef, pork, and lamb, and of brain and liver from old cows and sows was determined. Liver and kidney were found to be the richest source of the vitamin, and the meats of beef organs were higher in vitamin B_{12} potency than those of pork. Pancreas was found to contain considerable vitamin B_{12} activity after alkali treatment when assayed with *L. leichmannii,* but not when assayed with *E. gracilis.* Limitations in the use of alkali lability as an index of the true vitamin B_{12} potency of samples are discussed.

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A COMPARISON OF TWO STOCK RATIONS FOR ALBINO RATS

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Nearly 20 years ago, Mendel and Hubbell ('35) described the procedures that had been developed during the preceding 25 years for the maintenance of an albino rat colony at the Connecticut Agricultural Experiment Station. At that time a new colony diet had been in use for about 4 years and attention was directed to an increased rate of growth and a better reproductive performance. For example, following the change in type of food, the percentage of rats that produced young increased from 68 for the year 1925 to 93 in 1935, the number of young born per litter from 6.4 to 9.6, the litters weaned from 76 to 90% and the weaning weights at 21 days from 31 to 48 gm for male and from 30 to 47 gm for female rats.

Throughout the period since that report appeared, the colony has been maintained on essentially the same dietary regime that was initiated in 1931, so that data are now available from many generations of animals. In addition, information has been obtained concerning the growth and reproductive behavior of rats of the same strain but with the use of the well-known Bills modification of the Steenbock stock diet (Bills et al., '31). When the laboratory undertook the routine assay of vitamin D in milk in 1935 it was found impossible to produce rachitic rats for this assay with the use of the regular colony diet. Because a long-term study of breeding and lactation in rats fed this ration was considered

desirable, the following procedure was adopted and has been continued up to the present time: the main portion of the colony has been fed a supplemented calf meal diet, which will be referred to as the stock ration. At weaning, some rats are assigned to this group to perpetuate the colony, while others are assigned to the group fed the Bills diet. The latter are used exclusively to supply animals for the vitamin D assays, and never for replenishment of the colony. Thus, from the time this procedure was adopted, there have been two parallel groups of breeders, each carried through two or three reproductive cycles, but with different foods. In the case of the group which was fed the stock ration, the effects of breeding for many generations with no essential modification in food have been recorded, whereas in the case of the group fed the Bills diet, it has been possible to observe repeatedly the immediate effects of a change in type of food.

The G. L. F. Calf Meal which forms the major part of the stock ration has remained nearly constant in composition during more than 20 years of use in this laboratory. However, there have been a few changes, mainly the addition of sources of vitamins A and D. The current formula is given in table 1. A "paste food" supplied to nursing mothers and to young rats for 10 days after weaning now has the following composition: casein 25, whole milk powder 25, wheat germ 20, lard 24.5, corn starch 5 and cod liver oil (400 D) 0.5%. Both calf meal and "paste food" are supplied ad libitum. In addition each rat also receives 1 gm of dried yeast daily except Sunday and those without paste food are given 3 gm of wheat germ per week. No "green food" of any sort is ever used.

The general colony management has been similar to that described before (Mendel and Hubbell '35). Rats are first bred at 120 days of age, with three females and one male to a cage. Brother and sister matings are avoided and a female is seldom mated with the same male more than once. Each female is permitted three matings, with an interval of about three weeks between the time of weaning and subsequent

remating. All litters are reduced at birth to 8 young, with 4 of each sex whenever possible, and are weaned at 21 days of age, when the weights of the mother and of the individual young are recorded.

The same procedure has been used for the rats fed the Bills diet, except that after June 1945 the number of animals re-

TABLE 1

Composition of calf meal [1]

INGREDIENTS	WEIGHT
	lb.
Yellow corn meal	416
Linseed oil meal	300
Ground malt barley	200
Wheat red dog	440
Oat flour	300
Dried skim milk	260
Soluble blood flour	40
Irradiated yeast	0.5
Ground limestone	5
Iodized salt	20
Vitamin A feeding oil	3.5
Dicalcium phosphate	15
	2000
2 gm cobalt sulfate added	

[1] Cooperative Grange League Federation Exchange, Inc., Ithaca, N. Y.

TABLE 2

Summary of reproduction and lactation in albino rats

A comparison of two stock rations

YEAR	FOOD	NUMBER OF FEMALES MATED	PERCENTAGE OF FERTILE MATINGS	NUMBER OF YOUNG BORN PER LITTER	PERCENTAGE OF LITTERS WEANED	WEIGHTS AT WEANING	
						Males	Females
						gm	*gm*
1935	Stock [1]	104	93	9.6	90	48	47
1938–44	Stock	1755	79.8	9.3	89.5	48.1	46.6
	Bills [2]	2329	74.9	9.5	89.2	39.2	38.0
1945–52	Stock	1963	82.8	9.6	82.1	49.3	48.0
	Bills	3336	73.1	9.8	80.9	38.5	37.3

[1] Supplemented calf meal.

[2] Bills et al. ('31).

TABLE 3

Summary of the weights of nursing rats and their young, grouped according to litter sequence and to the number of young [1]

1945–52

FOOD	MATING	YOUNG PER LITTER	4-DAY WEIGHTS		17-DAY WEIGHTS		21-DAY WEIGHTS	
			Mothers	Young	Mothers	Young	Mothers	Young
			gm	*gm*	*gm*	*gm*	*gm*	*gm*
Stock	1	7–8	317 (279)	9.5 (2155)	327 (271)	35.8 (2076)	318 (271)	47.5 (2069)
		3–6	303 (71)	9.0 (366)	318 (80)	35.3 (413)	322 (81)	48.0 (416)
	2	7–8	344 (300)	9.9 (2364)	349 (296)	36.6 (2287)	346 (293)	49.2 (2281)
		3–6	347 (52)	9.8 (246)	354 (56)	37.3 (300)	340 (59)	51.4 (298)
	3	7–8	367 (219)	9.9 (1687)	379 (207)	37.8 (1593)	367 (205)	50.5 (1572)
		3–6	353 (40)	10.1 (201)	333 (50)	39.4 (253)	345 (50)	51.9 (260)
Bills	1	9–10	340 (310)	8.9 (2967)	323 (281)	26.8 (2676)	302 (278)	35.0 (2643)
		7–8	345 (182)	8.9 (1391)	325 (188)	28.2 (1438)	312 (191)	37.6 (1461)
		3–6	325 (83)	9.2 (447)	338 (106)	29.9 (564)	333 (105)	41.4 (554)
	2	9–10	373 (334)	9.3 (3212)	355 (312)	28.6 (2980)	330 (304)	37.4 (2915)
		7–8	374 (149)	9.4 (1129)	361 (152)	30.6 (1156)	344 (158)	40.8 (1202)
		3–6	375 (72)	9.2 (380)	377 (95)	32.5 (489)	361 (98)	43.4 (500)
	3	9–10	394 (226)	9.4 (2159)	374 (208)	29.5 (1980)	332 (205)	37.8 (1950)
		7–8	391 (117)	9.5 (895)	381 (122)	31.5 (936)	363 (127)	41.0 (971)
		3–6	392 (82)	9.3 (443)	378 (93)	33.0 (485)	403 (94)	44.4 (497)

[1] The number of animals in each group is given in parentheses. The young at 4 days and at 17 days were weighed as a group. The young at 21 days were weighed individually.

tained in each litter was increased to 9 or 10, whenever possible. This was done in an attempt to offset losses that occur during the nursing period. Table 2 shows a comparison of reproduction and lactation as indicated by the number of litters born and weaned and by the weights of the young at weaning for the entire period from 1938 through 1952. Inasmuch as after 1945 the data for the rats of the Bills group would reflect the fact that many litters contained 9 or 10 young, all data for each group have been given in two parts, one for the period from 1938 through 1944 and the other for the succeeding 8 years.

Some years ago, Greenwood ('40) called attention to the well-known fact that nursing rats start to eat the stock ration several days before they are weaned. For that reason it was suggested that the change in weight of the mother and also of the young during the period from the 4th to the 17th day of lactation might be a better measure of the effectiveness of a ration than the weaning weights, the criterion used by Smith et al. ('38). Consequently, since 1945, data have been accumulated to secure information on this point. Weights have been obtained routinely for both mothers and young at the end of 4, 17 and 21 days of lactation. The young were weighed as a group at 4 and at 17 days; the 21-day weights are the averages of the individual weights obtained when the litters were weaned. These data are summarized in table 3.

DISCUSSION

In the interpretation of these two summaries it should be understood that table 2 contains a record of the behavior of *all* females mated during the 15-year period. Table 3 concerns only those females that successfully weaned two or three litters. In the experiments of Smith et al. ('38), a study was made of 4 small groups of rats and their progeny during 4 matings in each of 7 generations, and the young for each new generation were always selected from 4th litters. In the management of a stock colony, such a procedure is not feasible and replacements in breeding stock have been taken from

any litters in which the young were healthy and of average weight. A large proportion of these replacements have come from litters in which at least 8 animals were born and 7 or 8 weaned.

It will be seen from table 2 that reproduction in the stock colony has been somewhat less satisfactory over the longer period than it was for the small number of animals included in the 1935 report. Slightly more than 80% of the females produced litters in contrast to 93% in 1935. Likewise, the proportion of litters weaned has declined in recent years from about 90% in the first two periods (1935 and 1938-1944) to 82% for the average value of the last 8 years. On the other hand, the weaning weights of the young have shown a slight increase during the 15-year period. The downward trend in the number of litters weaned had been evident in the annual summaries from which the data in table 2 are taken. Consequently Hubbell and Krehl ('52) attempted to determine whether the addition of either whole liver substance or orange juice to the stock ration would improve lactation. A small group of animals was carried through the F_4 generation using progeny of first matings for each succeeding generation. Both in respect to fertility and percentage of litters weaned, the animals of this group were inferior to those from first matings in the main colony during the same period.

In the group of rats fed the Bills ration, fertility was lower during each period than it was for the stock animals, being 74.9% for the first period and 73.1% for the second. The behavior in respect to the number of litters weaned was similar to that for stock animals, namely a drop from 89.2 to 80.9%. The weaning weights as recorded in the second period were somewhat lower than in the first, but the difference is small and probably not significant, because litters of 9 and 10 young are included. More than 50% of the mothers weaned these larger litters, and, as will be seen in table 3, the weaning weights of these rats are much lower than they are in litters of 8 or fewer. It would thus appear that while the stock ration is slightly more favorable for gestation, the ability to nurse

a litter is equally good when the Bills ration is employed, if the number of litters weaned is used as the measure of lactation success. If, however, the weights of the young at weaning are also considered, there are significant differences between the two groups. Weaning weights of rats in the stock groups are 9 to 10 gm above those in the Bills groups. That this difference may be attributed in part to the use of "paste food" in addition to calf meal has been demonstrated by unpublished experiments in which the Bills diet was also supplemented with "paste food," with a resulting marked increase in the weights of the young at weaning. Also, if calf meal is used without the "paste food" supplement, the weaning weights are comparable to those obtained with the Bills diet alone.

The lower rate of growth of animals fed the Bills ration is confined to the lactation period. During the weeks following weaning, rats in the Bills group become consistently heavier than the stock animals. At 120 days of age, just before the first mating, the average weight of stock males is about 440 gm, in the Bills group the weight is 500 gm or more. Older adult males in the former group are nearly always 100 to 150 gm lighter than corresponding animals in the Bills group. That lactating females tend to be heavier when the Bills diet is used can be seen from the summaries in table 3, which presents a comparison of the weights of both mothers and nursing young for rats fed each diet, and for each of three matings. Although data in this table are recorded for all mothers that weaned at least two litters, the discussion will be confined chiefly to those cases where there were 7 or 8 young in a litter.

For the stock rats it will be seen that the weights of the mothers were remarkably constant during lactation. Slight gains in weight were noted in 5 out of 6 groups for the weights recorded at 17 days, but, at weaning, the weights were the same as they were after the first 4 days of nursing. It is evident that the food has been amply adequate to maintain the weight of the mother while the young were gaining a total

of about 300 gm. In the case of the Bills rats the picture is quite different. There were marked losses in the weights of the mothers between 4 and 17 days and also between 17 days and weaning. The total loss in the first lactation was 33 gm and in the third 28 gm. During this same period the young gained about 70 gm less than corresponding rats in stock litters. If the weight of the mother following either 17 or 21 days of nursing a litter is to be used as a measure of the effectiveness of the ration for lactation, it would seem that, for this colony at least, the weight at weaning, which is obtained routinely, is entirely satisfactory. Even though the young have started to eat some of the food, there is a decrease in the weight of the mother between 17 and 21 days of lactation. Although this decline is almost negligible for the stock rats, in the Bills group it is as great during these last 4 days as it is for the earlier period of almost two weeks. On whichever basis the efficiency of the food is measured, it is evident that the mothers in the Bills group have been subjected to a greater drain during lactation than the stock animals.

A further indication of the marked depletion in the mother when the Bills diet is used may be seen in her change in weight during the first few days after weaning. It would be difficult to give exact figures, because the interval between weaning and the first regular weighing thereafter is not always the same. However, it has been observed repeatedly that there tends to be a sharp rise in the weight of the mother as soon as the litter has been weaned. On the other hand, the weights of the stock animals show very slight changes under comparable conditions.

Some mention should be made of the influence of the number of rats per litter and of the position in the litter series on the weights of the mother and young. In general the tendency is in the direction to be expected, namely that in the Bills group a mother who is nursing a litter of 9 or 10 rats loses more weight than one with a smaller litter. This loss is greater for second and third matings than when first lit-

ters are being nursed. The young themselves are from 2.6 to 3.4 gm lighter at weaning when there are 9 or 10 in the litter in comparison with the weights for litters of 7 or 8 which have been taken as a standard. If a mother in the Bills group is nursing a litter of less than 7, she is able to maintain her weight and even gain slightly, and, as would be expected, the young are from 6.0 to 6.6 gm heavier than those in a litter of 9 or 10.

In connection with the effect of the number of young per litter on the general condition of the mother or their own weights at weaning, it is of interest to recall a statement made by Bills ('47) that "in raising rats for the line test, it is well to reduce the number of young in large litters (more than 8) to 6. This lightens the burden of nursing on the mothers subjected to heaviest reproduction, and makes for uniformity of weight in the young." Insofar as there is less drain on the mother if she is nursing a small litter, our experience during the last 15 years is similar to that indicated by Bills. However, we have found that rats in the larger litters are very uniform in weight, not only within litters, but also between litters. Inasmuch as large numbers of animals have been needed for the assays of vitamin D in milk, the procedure we have followed, of saving 9 or 10 young whenever possible, has proved to be feasible.

SUMMARY

Data have been assembled from the breeding records of a colony of albino rats over a period of 15 years. The effects of two stock rations, our long-used supplemented calf meal diet and the Bills modification of the Steenbock stock diet, have been compared with respect to the percentage of litters cast and weaned and the weights of both mothers and young at three intervals during the lactation period. Lactation has been somewhat less satisfactory in recent years with each ration. With the first, a nursing mother has been able to maintain her weight until the young were weaned, regardless of the size of the litter. With the second, the mother lost

weight throughout lactation, unless the litter was very small (3–6). The weaning weights of the young in each group varied with the number of young in a litter and with the position in the litter series.

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BENEFICIAL EFFECT OF LOW-FAT DIETS ON THE SWIMMING PERFORMANCE OF RATS AND MICE IN COLD WATER[1]

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Considerable data are available concerning the deleterious effects of diets low in fat and the protective effects of high fat rations on animals exposed to various stressor agents (Burr, '42; Deuel and Greenberg, '50; Ershoff, '53; Bosshardt and Huff, '53). The findings of Deuel and co-workers (Deuel et al., '47; Scheer et al., '47) are of particular pertinence in this regard. These workers found that rats maintained on high fat diets swam significantly longer at a water temperature of 36–38°C. than those on a low-fat ration. In the present communication, however, data are presented indicating that when tests were conducted at a water temperature of 20°C., the swimming performance of rats and mice on a low-fat ration was significantly longer than that of animals fed a similar diet supplemented with fat.

PROCEDURE AND RESULTS

Experiments on young mice

Diets. Two basal rations were employed in the present experiments: diet A and diet B. Diet A was a low-fat ration of the following composition in percent: dextrose, 69.5; casein,[2]

[1] Acknowledgement is made to the Office of Naval Research for partial support of this investigation. Communication no. 360 from the Department of Biochemistry and Nutrition, University of Southern California.

[2] Vitamin-free test casein, General Biochemicals Co., Inc., Chagrin Falls, Ohio.

24; salt mixture,[3] 4.5; and cellulose,[4] 2. To each kilogram of the above were added the following synthetic vitamins: thiamine hydrochloride, 10 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; 2-methyl-naphthoquinone, 5 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 100 μg; and choline chloride, 2 mg. In addition to the above each mouse received twice weekly an oral supplement of 0.5 mg alpha-tocopherol acetate and a vitamin A and D concentrate[5] containing 20 U.S.P. units of vitamin A and 2 U.S.P. units of vitamin D. Diet B was similar in composition but contained 10% cottonseed oil[6] added in place of an equal amount of dextrose. Sixty male mice of the Webster strain were selected at 11 to 14 gm in weight and were fed the above diets ad libitum for 21 days (30 animals per group). The mice were placed in metal cages with raised screen bottoms to minimize access to feces and were fed daily. All food not consumed 24 hours after feeding was discarded.

Method of test. After 21 days of feeding the average weight increment of mice on diets A and B was 8.8 gm and 9.1 gm respectively. Swimming tests were conducted at this time on all animals. The procedure employed was as follows: mice were placed in a bucket with smooth vertical sides or a large wide-mouthed glass jar filled to a depth of 10 inches with water. Tests were conducted at a water temperature of both 37° and 20°C. Twelve mice from each dietary group were tested at the higher temperature; the remainder (18 animals per group) at the lower. Measurements were made of the length of time that elapsed before an animal's head remained

[3] Hubbell, Mendel, and Wakeman Salt Mixture, General Biochemicals Co., Inc., Chagrin Falls, Ohio.

[4] Solka Floc, Brown and Co., Boston, Mass.

[5] NOPCO WDA (a water-dispersable vitamins A and D concentrate from fish liver oils), containing 62,000 U.S.P. units of vitamin A and 6,200 U.S.P. units of vitamin D per gram, National Oil Products Co., Harrison, New Jersey.

[6] Wesson.

submerged below the surface of the water for a period of 30 seconds[7].

Results. No significant difference was observed in the swimming performance of mice on the two diets at a water temperature of 37°C. All of the mice in each dietary group survived a test period of 4 hours at the end of which they were still swimming. When tests were conducted at a water temperature of 20°C., however, a significant difference was observed between the two dietary groups. Mice on the fat-supplemented ration (diet B) swam an average of 11.7 minutes (range 5 to 21 minutes); only 4 of the 18 mice tested on the fat-free ration (diet A), however, swam for less than 4 hours (44, 65, 118 and 224 minutes); all other mice in this group were still swimming at the end of 240 minutes at which time the test was discontinued.

EXPERIMENTS IN ADULT MICE AT 20°C.

Experiments were next conducted to determine the comparative effects of a fat-free diet and a fat-supplemented diet on the swimming performance of adult mice at a water temperature of 20°C. Twelve male mice of the Webster strain which had been raised from weaning on a natural food ration were selected for the following experiment at approximately 4 months of age and a body weight of 28 to 34 gm. Half the animals were fed the basal fat-free ration (diet A); the remaining animals were administered the fat-supplemented ration (diet B). After 21 days of ad libitum feeding the average weight increment of mice on diets A and B was 2.9 gm and 3.3 gm respectively. Swimming tests were conducted at this time at a water temperature of 20°C. as previously described. Mice on the fat-supplemented ration (diet B) swam an average of 14.5 minutes (range, 7 to 24 minutes); three of the mice on the fat-free ration (diet A) swam for 31, 40

[7] Although most animals drowned if the head remained submerged for 15 to 20 seconds, an occasional animal rose to the surface after remaining submerged for this period and continued its swim. The end point of 30 seconds was selected because every animal whose head remained submerged for this period had drowned.

and 108 minutes; the remaining three animals in this group were still swimming at the end of 240 minutes at which time the test was discontinued. It is apparent from these findings that the beneficial effect of a fat-free diet on the swimming capacity of mice in cold water could be demonstrated not only in mice that were started on this diet at the time of weaning but also in animals that were raised to maturity on a natural-food ration.

Tests were next conducted to determine the comparative effects of a fat-free diet and similar rations containing graded levels of fat on the swimming performance of mice at a water temperature of 20°C. Fifty male mice of the Webster strain were selected at 11 to 14 gm in weight and were fed the following 5 diets (10 animals per group): (a) diet A; (b) diet A plus 1% cottonseed oil; (c) diet A plus 2.5% cottonseed oil; (d) diet A plus 10% cottonseed oil; and (e) diet A plus 10% hydrogenated coconut oil.[8] The oil supplements were incorporated in the basal ration in place of an equal amount of dextrose. The animals were fed the above diets ad libitum under the experimental conditions indicated in experiment 1. After 18 days of feeding swimming tests were conducted with all mice at a water temperature of 20°C. Results are summarized in table 1.

Findings indicate that the duration of the swim was inversely proportional to the cottonseed oil content of the diet. On the fat-free ration the average swimming time was in excess of 90 minutes with 7 of the 10 animals in this group still swimming at the end of two hours at which time the test was terminated. On the 1% cottonseed oil ration, the average swimming time was 52 minutes with two animals still swimming at the termination of the experiment. Mice receiving diets containing 2.5% and 10% cottonseed oil swam 17.9 and 11.3 minutes respectively with all mice drowning within 30 minutes. The average swimming time on the ration containing 10% hydrogenated coconut oil (21.1 minutes) was almost twice as long as that obtained on the diet containing 10%

[8] Hydrogenated Deodorized Coconut Oil, Best Foods, Inc., New York, N. Y.

cottonseed oil. The difference between these two groups appeared to be statistically significant.

Experiments were next conducted to determine the minimum length of time required for a fat-free diet to exert a protective effect on the swimming performance of mice at a water temperature of 20°C. In the first experiment 20 male

TABLE 1

Comparative effects of a fat-free diet and similar rations containing graded levels of fat on the swimming performance of mice at a water temperature of 20°C. (10 animals per group)

SUPPLEMENTS ADDED TO BASAL RATION	DURATION OF SWIM	AVERAGE SWIMMING TIME [1,2]
	minutes	*minutes*
None	18,21,23,120+,120+,120+,120+,120+,120+,120+	90.2+
1% Cottonseed oil	13,14,19,27,28,35,59,88,120+,120+	52.3+
2.5% Cottonseed oil	14,15,16,17,17,18,18,19,22,23	17.9±0.9 [2]
10.% Cottonseed oil	9,10,10,11,11,11,12,12,13,14	11.3±0.5 [2]
10% Hydrogenated coconut oil	13,13,13,13,15,17,30,31,32,34	21.1±2.8 [2]

[1] Data were calculated on the basis of a 120 minute swimming performance for animals still swimming at the termination of the experiment.

[2] Including standard error of the mean calculated as follows: $\frac{\sqrt{\frac{\Sigma d^2}{n}}}{\sqrt{n}}$ where "d" is the deviation from the mean and "n" is the number of observations.

mice of the Webster strain were selected at 11 to 14 gm in weight and were fed diets A and B (10 animals per group) under conditions similar to those previously described. After 11 days of feeding swimming tests were conducted with all mice at a water temperature of 20°C. All mice fed diet B drowned within 20 mintues (average swimming time, 12.4 minutes). With the exception of two mice which swam for 35 and 45 minutes, all the mice on diet A were still swimming

after 7 hours and 20 minutes at which time the test was discontinued.

Experiments similar to the above were subsequently conducted in which animals were allowed to swim after three and 5 days of feeding (10 animals per group on each diet). On diet B the average swimming time both after three days and 5 days of feeding was less than 12 minutes. On diet A after three days of feeding, the average swimming time was 78.7 minutes with only two out of 10 animals still swimming after two hours at which time the test was discontinued. After 5 days of feeding, however, the average swimming time of mice on diet A was 84.6 minutes with 6 out of the 10 animals in this group still swimming at the termination of the test after two hours. It is apparent from these findings that the protective effect of the fat-free diet is manifest within three days of feeding but that the optimal effect does not occur until somewhat later (5th to 11th day).

EXPERIMENTS ON RATS

Diets and methods. The basal low-fat ration employed in the following experiment consisted of sucrose, 71% casein, 24%; salt mixture, 5%; and the following synthetic vitamins per kilogram of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid. 60 mg; biotin, 4 mg; folic acid, 10 mg; 2-methylnaphthoquinone, 5 mg; ascorbic acid, 200 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 100 μg; and choline chloride, 2 mg. To each kilogram of diet were also added 4,000 U.S.P. units of vitamin A and 400 U.S.P. units of vitamin D. Each rat also received once weekly a supplement of 4.5 mg of alpha-tocopherol acetate. One hundred and five female rats of the Wistar strain were selected at 21 to 23 days of age and at a body weight of 36 to 45 gm for the present experiment. Animals were kept in metal cages with raised screen bottoms to minimize acccess to feces and were fed ad libitum the following 7 diets (15 animals per group): (a) basal ration alone; (b)

basal ration plus 1% cottonseed oil; (c) basal ration plus 2.5% cottonseed oil; (d) basal ration plus 10% cottonseed oil; (e) basal ration plus 1% hydrogenated coconut oil; (f) basal ration plus 2.5% hydrogenated coconut oil; and (g) basal ration plus 10% hydrogenated coconut oil. The oil supplements were incorporated in the basal ration in place of an equal amount of sucrose. Diets were made up weekly and stored under refrigeration when not in use. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet.

After 28 days of feeding at which time the average body weight for rats in the various groups ranged between 110 and 119 gm, swimming tests were conducted on all animals. The procedure employed was as follows: Rats were placed in a barrel approximately 33 inches in height and 28 inches in maximum diameter, with smooth vertical sides, and filled to a depth of 18 inches with water. Tests were conducted at a water temperature of both 37°C. and 20°C. Six rats from each dietary group were tested at the higher temperature; the remainder (9 animals per group) at the lower. Measurements were made of the length of time that elapsed before an animal's head remained submerged below the surface of the water for a period of 30 seconds.[9]

Results. No significant difference was observed in the swimming performance of rats on the various dietary regimes at a water temperature of 37°C. All the rats in each dietary group survived a test period of 4 hours at the end of which they were still swimming. When tests were conducted at a water temperature of 20°C., however, a significant difference was observed between the various dietary groups (table 2).[10] Rats fed the basal ration supplemented with either 10% cottonseed oil or hydrogenated coconut oil swam an average of

[9] See footnote 7, page 441.

[10] That differences between the various groups were statistically significant is indicated by the fact that the longest swimming time on any of the diets containing either 10% or 2.5% of cottonseed oil or hydrogenated coconut oil was less than the average for rats fed the fat-free diet.

11.2 and 12.7 minutes respectively. The average duration of the swim was practically doubled when the fat content of the ration was reduced to 2.5% (of either cottonseed oil or hydrogenated coconut oil). The duration of the swim was further increased when the fat content of the ration was reduced to 1% (of either cottonseed oil or hydrogenated coconut oil) or when the fat in the ration was eliminated in entirety. No significant difference was observed between the swimming performance of rats on the fat-free basal ration and animals

TABLE 2

Effects of a fat-free diet and of graded levels of cottonseed oil and hydrogenated coconut oil on the swimming performance of rats at a water temperature of 20°C. (9 animals per group)

SUPPLEMENTS ADDED TO BASAL RATION	DURATION OF SWIM	
	Average	Range
	minutes	
None	51.1	(20–148)
1% Cottonseed oil	45.0	(12–106)
1% Hydrogenated coconut oil	36.8	(21–103)
2.5% Cottonseed oil	24.0	(12– 34)
2.5% Hydrogenated coconut oil	22.1	(11– 47)
10% Cottonseed oil	11.2	(7– 15)
10% Hydrogenated coconut oil	12.7	(9– 17)

on a similar ration supplemeted with 1% cottonseed oil or hydrogenated coconut oil.

Similar tests were subsequently conducted on 20 female rats of the Long-Evans strain which were selected at weaning and were fed for 4 weeks on the basal fat-free ration and a similar diet supplemented with 10% cottonseed oil (10 animals per group). At a water temperature of 20°C., rats on the latter diet swam an average of 14.5 minutes (range 13 to 18 minutes); rats on the basal fat-free ration, however, swam for 33.7 minutes (range 21 to 55 minutes). It is apparent from these findings that Long-Evans rats as well as Wistar rats

demonstrated the protective effect of a low-fat ration on the swimming performance of rats at a water temperature of 20°C.

DISCUSSION

Present findings indicate that in both rats and mice the swimming performance of animals at a water temperature of 20°C. was inversely proportional to the fat content of the diet. Animals fed fat-free diets or diets containing 1% cottonseed oil or 1% hydrogenated coconut oil swam significantly longer than animals fed similar rations supplemented with 10% cottonseed oil or 10% hydrogenated coconut oil. Intermediate results were obtained at a 2.5% fat level. The protective effect of low-fat rations was more marked in mice than in rats, but the results were statistically significant in both species. The protective effect of the low-fat ration in mice was manifest within three days of feeding. When swimming tests were conducted at a water temperature of 37°C., no significant difference was observed between the various dietary groups.

No data are available to indicate what factors are responsible for the protective effect of low-fat diets on the swimming performance of rats and mice at a water temperature of 20°C. Findings do not appear to be due to a deficiency of "essential fatty acids" inasmuch as hydrogenated coconut oil (which contains virtually none of these acids) was effective in reducing the swimming time of both rats and mice on a fat-free diet. Furthermore, it is unlikely that a deficiency of "essential fatty acids" could possibly result in the short period of time required for the fat-free diet to exert its protective effect. In general animals on the fat-free diet were more buoyant than those ingesting diets containing higher levels of fat. Differences in specific gravity induced by the various diets might accordingly be suspected as having contributed to the diverse results. The addition of one gram weights to mice on the fat-free diets (approximately 5% of their body weight), however, did not significantly impair their swimming performance at a water temperature of 20°C. Furthermore, if differences in specific gravity did exist between animals on

the various diets, they were not of sufficient import to impair the swimming performance of these animals at a water temperature of 37°C. A more probable cause of the differences in swimming capacity at a water temperature of 20°C. is differences in the vital capacity of animals on the various diets. Lanoue ('52) has observed that it becomes increasingly difficult for persons to take adequate breaths when immersed in cold water. Further studies are indicated to determine to what extent possible differences in the vital capacity of animals on the various diets at a water temperature of 20°C. were responsible for the observed results.

Present findings differ from those reported by Deuel and co-workers (Deuel et al., '47; Scheer et al., '47) who found that rats maintained on high fat diets swam significantly longer at a water temperature of 36–38°C., than animals on a low fat ration. The experimental conditions in the present experiment differed considerably, however, from those employed by Deuel et al. In the latter studies, work (induced by adding weights to the rat at graded intervals) was employed as the stressor agent. In the present experiment the stressor agent was not work *per se* but, at least in part, the stress of immersion in cold water and the attendant loss in body temperature. It is not readily apparent what factors were responsible for the diverse effects of low fat diets in the experiments indicated above. It would appear from those findings that a diet which might be superior in protecting against one type of stressor agent might actually be inferior in protecting against another.

SUMMARY

The swimming performance of rats and mice at a water temperature of 20°C. was inversely proportional to the fat content of the diet. Animals fed purified fat-free diets or similar diets supplemented with 1% cottonseed oil or 1% hydrogenated coconut oil swam significantly longer than animals fed similar rations supplemented with 10% cottonseed oil or 10% hydrogenated coconut oil. Intermediate results

were obtained at a 2.5% level. The protective effect of low fat rations in mice was manifest within three days of feeding. When swimming tests were conducted at a water temperature of 37°C., no significant difference in swimming performance occurred between the various dietary groups.

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EFFECTS OF DIETARY STEROLS AND STEROL ESTERS ON PLASMA AND LIVER CHOLESTEROL IN THE CHICK [1]

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ONE FIGURE

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In recent studies it has been shown that the hypercholesteremia and excessive deposition of liver cholesterol produced in chickens by high cholesterol intake can be prevented by simultaneous feeding of certain poorly absorbed sterols — mixed soy sterols, mixed sitosterols (Peterson, '51; Peterson et al., '52), β-sitosterol, stigmasterol, ergosterol (Peterson et al., '53), and dihydrocholesterol (Siperstein et al., '53).

The chemical form in which the sterols are present in the diet profoundly affects the tissue levels of cholesterol. Thus when mixed soy sterols were esterified with capric acid they no longer controlled hypercholesteremia and deposition of tissue cholesterol when fed with cholesterol. Cholesterol itself fed as the caprate ester did not produce hypercholesteremia (Peterson et al., '53).

The present report concerns further experiments on the relationship of dietary sterols or their esters to tissue levels of cholesterol.

METHODS

The basal diet and experimental procedures employed were the same as those described previously (Peterson et al., '52,

[1] This study was supported by a grant-in-aid from the American Heart Association.

'53). Male Single Comb White Leghorn chicks were kept on the laboratory stock diet until the age of three weeks, when they were placed on the experimental diets. Plasmas were analyzed weekly and livers terminally for cholesterol by the method of Sperry and Webb ('50). Presence of 7-dehydrocholesterol or ergosterol was estimated by the Liebermann-Burchard "fast acting" sterol method of Moore and Baumann ('52).

Sterol esters were prepared by the procedure of Cataline et al. ('44). Characteristics of the sterol esters are shown in table 1.

TABLE 1

Characteristics of sterol esters

COMPOUND	OPTICAL ROTATION ($CHCl_3$) EXP.	OPTICAL ROTATION ($CHCl_3$) LIT.[1]	MELTING POINT EXP.	MELTING POINT LIT.[1]	% FREE STEROL
Cholesteryl acetate	— 44.8°	— 47.4°	115°	115°	...
Cholesteryl caprate	— 30.2°	— 29.8°	92°	93°	0.34
Cholesteryl myristate	— 27.2°	— 26.6°	86°	86°	1.01
Cholesteryl palmitate	— 22.5°	— 25.1°	86–87.5°	90°	0.11
Cholesteryl stearate	— 24.1°	— 24.3°	82°	82.5°	0.10
Cholesteryl oleate	— 21.6°	— 23.3°	44–45°	44.5°	0.31
Dihydrocholesteryl palmitate	— 8.3°	...	93°	..	< 0.1
Ergosteryl palmitate	— 50.4°	— 51°	107–108°	107–108°	< 0.1

[1] Deuel, H. J., Jr., The Lipids, their chemistry and biochemistry. Volume 1, 1951.

EXPERIMENTAL AND RESULTS

Cholesterol esters

The following esters of cholesterol were fed in the diets as indicated in figure 1: acetate, caprate, myristate, palmitate, stearate and oleate. Each was incorporated in the diet at a level equivalent to 1% of cholesterol. Four per cent cottonseed oil was added to each diet.

Plasma cholesterol rose to the highest levels in those birds fed free cholesterol. Among the birds fed the esters the degree of cholesteremia decreased in the following order: acetate, oleate, caprate, myristate, palmitate, stearate (fig. 1). The plasma level of esterified cholesterol averaged about one-fourth of the total cholesterol in all groups. In birds fed the palmitate and stearate esters the extent of cholesteremia did not differ greatly from that of birds on the basal

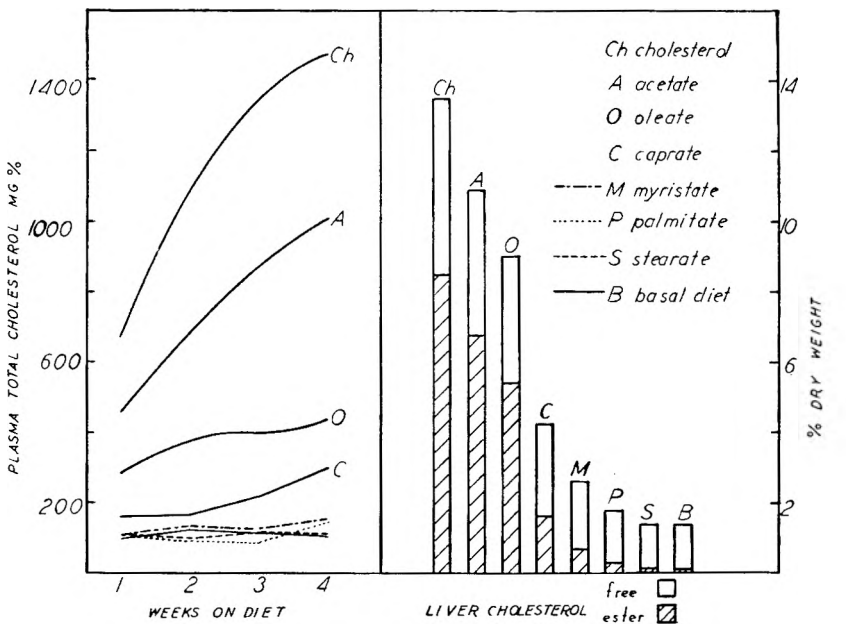

Fig. 1 Effects of dietary sterol esters on tissue cholesterol.

(low cholesterol) diet. Liver levels of cholesterol decreased in the same order as plasma cholesterol. Where the deposits of liver cholesterol were very great as in those animals fed free cholesterol, cholesteryl acetate, and cholesteryl oleate, most of this cholesterol was esterified. Where the liver cholesterol levels did not differ greatly from normal, most of the cholesterol was in the free state.

Although the cholesterol from cholesteryl palmitate is itself apparently not well absorbed this ester did not depress

plasma levels of cholesterol when fed with free cholesterol. Liver levels of cholesterol appeared to be slightly lower when a mixture of cholesterol and cholesteryl palmitate was fed than when cholesterol alone was fed (table 2).

TABLE 2

Effects of sterols and sterol esters on tissue cholesterol levels

(Average values, 5 chicks per group — on diet 3 weeks)

DIET SUPPLEMENT [1]	PLASMA			LIVER			
	Cholesterol		Fast acting sterol	Cholesterol		Fast acting sterol	
	Total	Free	Total	Total	Free	Total	Free
	mg %	*mg %*	*mg %*	*% dry wt.*		*% dry wt.*	
None (Basal diet)	95	18	< 5	1.57	1.39	0.06	...
1% cholesterol	1164	329	< 5	17.1	6.17	0.31	...
1% cholesterol 2.4% ergosterol	184	33	< 5	4.62	2.68	0.10	...
1% cholesterol 2.4% ergosteryl palmitate	1077	251	< 5	11.5	4.49	0.12	...
1% cholesterol 1.5% dihydrocholesterol	151	44	..	4.20	2.34	0.06	...
1% cholesterol 2.4% dihydrocholesteryl palmitate	1025	294	..	14.1	4.96	0.09	...
2.4% cholesteryl palmitate	110	16	..	1.61	1.11	...	...
1% cholesterol 2.4% cholesteryl palmitate	1419	301	..	7.49	2.76	...	...
1% 7-dehydrocholesterol	213	...	227	1.60	1.03	2.90	1.81
1% cholesterol 1% 7-dehydrocholesterol	871	...	516	5.91	2.39	3.90	1.71

[1] Four per cent cottonseed oil added to all diets.

... not determined.

Other sterols

Esterification of ergosterol or of dihydrocholesterol with palmitic acid destroyed the ability to prevent increases in plasma and liver cholesterol which is exhibited by the free sterols (table 2). The plasma levels of "fast acting" sterols

TABLE 3

Effects of various dietary supplements on plasma cholesterol

(9 male chicks per group)

SUPPLEMENT	PLASMA CHOLESTEROL Total cholesterol	3RD WEEK ON DIET [1] Free cholesterol
	mg/100 ml	*mg/100 ml*
None	101	24
1% Cholesterol	631	168
1% Cholesterol 0.3% Cholic acid	1098	415
1% Cholesterol 0.3% Cholic acid 1.5% Soy sterols	293	89
1% Cholesterol 4% Oleic acid	1065	409
1% Cholesterol 4% Oleic acid 1.5% Soy sterols	222	66
1% Cholesterol 4% Cottonseed oil	1450	497
1% Cholesterol 4% Cottonseed oil 1.5% Soy sterols	195	54
1% Cholesterol 4% Palmitic acid	530	168

[1] The duration of the experiment was 6 weeks. Similar relative levels were found at each weekly sampling, but maximum levels were not reached until the third week.

(probably 7-dehydrocholesterol or ergosterol) were not influenced by the presence or absence of cholesterol or ergosterol in the diet, although there appeared to be a slight increase in liver 7-dehydrocholesterol when cholesterol was fed under conditions which produced hypercholesteremia. Tables 2 and 3 include analyses only for the terminal week of the experi-

ment. Similar results were obtained during the preceding weeks.

Feeding of 7-dehydrocholesterol produced marked increases in 7-dehydrocholesterol ("fast acting" sterols) of both plasma and liver and slight increases in cholesterol levels of these tissues. Although 7-dehydrocholesterol never increased to the high levels obtained with cholesterol, feeding a mixture of cholesterol and 7-dehydrocholesterol appeared to increase the absorption of 7-dehydrocholesterol and slightly decrease that of cholesterol (table 2).

Fat and cholic acid

Increases in plasma cholesterol beyond that obtained when cholesterol alone was included in the diet were found when cholic acid, cottonseed oil or oleic acid were added to the cholesterol diet, but not when palmitic acid was the supplement (table 3).

DISCUSSION

These data indicate that the chemical form in which sterols are present in the diet has a marked effect upon the absorption of cholesterol. Esterification of the hydroxyl group of cholesterol appears to decrease the rate of absorption. Among the various esters the highest plasma and liver cholesterol levels were obtained with the acetate; with oleate the levels were somewhat lower. Increase in the chain length of the saturated fatty acid esterified to cholesterol produced decreasing blood and liver levels. It is possible that the cholesterol levels obtained in these tissues reflect the degree of hydrolysis of the ester in the intestinal tract since free cholesterol produced the highest tissue levels of cholesterol.

The fact that the palmitate esters of such poorly absorbed sterols as ergosterol and dihydrocholesterol were not effective in preventing increases in tissue levels of cholesterol when high levels of cholesterol were fed, indicates that these compounds must have a free hydroxyl group in order to be effective inhibitors of cholesterol absorption. Cholesterol

palmitate itself was apparently poorly absorbed but did not inhibit rises in tissue levels of cholesterol when fed with cholesterol.

The plasma cholesterol levels obtained when cholesterol was fed without added fat (table 3) were somewhat higher than those obtained previously (Peterson et al., '53), but they were markedly increased by the addition to the diet of cottonseed oil, oleic acid, or cholic acid. Each of these marked rises could be inhibited by the presence of mixed soy sterols in the diet. Palmitic acid fed with cholesterol did not produce plasma levels greater than those obtained when cholesterol was fed without added fat. Cholic acid, cottonseed oil and oleic acid may have an indirect effect on cholesterol absorption by stimulating bile flow. The presence of bile has been shown to be essential for cholesterol absorption in the rat (Siperstein et al., '52).

During its absorption in the rat cholesterol is esterified (Bollman and Flock, '51; Chaikoff et al., '52). When C^{14}-labeled cholesterol was fed to rats with either mixed soy sterols or β-sitosterol, there was a pronounced decrease in cholesterol absorption; and of the cholesterol absorbed, there was a decrease in the esterified fraction (Hernandez et al., '53). In a study comparing the absorption of C^{14}-labeled cholesterol and C^{14}-labeled epicholesterol it was found that cholesterol was absorbed about twice as fast as epicholesterol. About 50% of the cholesterol absorbed was esterified whereas the epicholesterol absorbed was in the free form (Hernandez et al., '54). The authors suggest that esterification may be responsible for the differences in the rates of absorption of the two compounds. This would be consistent with the fact that dietary free cholesterol is more rapidly absorbed than cholesterol which is already present in the diet as esterified cholesterol. It has previously been suggested (Peterson et al., '53) that the plant sterols produce their inhibitory effect on cholesterol absorption by interfering with an esterifying mechanism. Cholesterol absorption is apparently somewhat depressed by the presence of 7-dehydrocholesterol, but this

substance evidently does not tie up the esterifying mechanism since it is itself absorbed to a considerable extent as compared, for example, with ergosterol where there is no evidence of an increase in the tissues when large amounts are fed (table 2).

SUMMARY

Feeding cholesterol esterified with fatty acids produced lower plasma and liver levels of cholesterol in young male chicks than did the feeding of free cholesterol. With cholesterol acetate this difference was slight but with some saturated fatty acid esters the difference was pronounced and the tissue levels decreased in the following order: caprate, myristate, palmitate, and stearate. With the last two esters plasma and liver cholesterol were approximately the same as those of normal chicks on low cholesterol diets. Cholesteryl oleate produced an effect intermediate between that of acetate and caprate. Although cholesteryl palmitate was apparently poorly absorbed it did not inhibit increases in tissue cholesterol when fed along with cholesterol.

The palmitates of ergosterol and dihydrocholesterol do not prevent increases in tissue cholesterol when fed with cholesterol as do the free sterols.

Tissue levels of 7-dehydrocholesterol were higher and those of cholesterol were lower when a mixture of these two sterols was fed when either was fed alone.

Tissue levels of cholesterol were increased by feeding cholic acid, oleic acid or cottonseed oil in combination with cholesterol. These increases were prevented by the presence of mixed soy sterols in the diet.

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USE OF UREA-INCLUSION COMPOUND CONTAINING ESSENTIAL FATTY ACID IN AN EXPERIMENTAL DIET [1]

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TWO FIGURES

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INTRODUCTION

When the fat incorporated in an experimental diet contains essential fatty acids, the danger always exists that undesirable rancidity may develop, and that essential nutrients may be destroyed, at least partially, by oxidation with atmospheric oxygen. Modern laboratory practice requires refrigeration of experimental diets to reduce this tendency, but, over long periods of time, mere refrigeration may not be enough to prevent autoxidation, particularly where the investigation directly concerns essential fatty acids.

Urea forms inclusion compounds with a variety of long chain normal aliphatic compounds. In the presence of such substances, urea crystallizes in a hexagonal lattice in which adequate space remains to harbor the "guest molecules." Long chain fatty acids and mono-esters form such urea-inclusion compounds easily, and unsaturated acids or esters are not subject to autoxidation or polymerization while held in such a complex (Schlenk and Holman, '50). Urea-inclusion compounds of fatty acids contain approximately 25% fatty acid. The preparation and properties of urea-inclusion com-

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pounds have been described in detail in a recent review by Schlenk ('54).

Since the discovery that urea-inclusion compounds of essential fatty acids or esters are not subject to autoxidation, they have been used in this laboratory as a means of incorporating unsaturated fatty acids in experimental diets. By this means the essential fatty acid can be administered steadily in the diet rather than by intermittent oral dosing. The urea-inclusion compound is stable as long as it remains dry, and the essential fatty acid is not released until the complex arrives in the gastrointestinal tract of the animal. Thus the investigator is assured that the animal receives essential fatty acids and not merely oxidation products derived therefrom, and that the dosage is spread more evenly throughout the period of supplementation.

The present report shows that essential fatty acids administered as inclusion compounds are still effective in curing fat deficiency. Measurement of the total arachidonic acid content of the rat carcass was chosen as the most direct criterion of essential fatty acid utilization (Widmer and Holman, '50). Considerable experience had indicated that urea-inclusion compounds containing essential fatty acids cured fat-deficient rats of their symptoms or increased the arachidonic acid content of the carcasses, but these effects had not been previously measured on the same group of animals.

EXPERIMENTAL

Each experimental group consisted of 8 4-month-old Sprague-Dawley female rats which had been placed on a fat-free diet at weaning age. The fat-free diet consisted of 20 parts vitamin-free casein, 72 parts sucrose, 4 parts Hubbell, Mendel and Wakeman salts, and 4 parts alphacel powdered cellulose.[2] To each kilogram of the above mixture the following vitamins were added: 450 mg ascorbic acid, 65 mg calcium pantothenate, 1.2 gm inositol, 5 mg 2-methyl-1,4-naph-

[2] Alphacel.

thoquinone, 30 mg niacin, 72 mg thiamin hydrochloride, 1.2 gm choline chloride, 12 mg folic acid and 4.5 μg biotin. To each kilogram of this basal ration the following fat-soluble vitamins were added: 5 mg beta carotene, 20 mμg calciferol and 100 mg alpha-tocopherol. Three experimental diets were prepared from the above, each containing in addition to the fat-soluble vitamins one of the following: (1) 80 gm corn oil ethyl ester urea-inclusion compound, (2) 20 gm corn oil, or (3) 60 gm urea per kilogram of basal diet. Diets 1 and 2 are approximately equivalent in linoleic acid content. These three diets plus a control diet containing only fat-soluble vitamins were stored in 2-kg batches (one week's supply) at — 16°C. These precautions were taken to minimize effects due to autoxidation.

The ethyl esters of corn oil were prepared by alkaline ethanolysis of corn oil by warming 250 ml absolute ethanol, in which 2.7 gm sodium had been dissolved, with each kilogram corn oil for three hours at 60 to 70°C. The ethyl esters were decanted from the glycerol layer, washed several times with hot distilled water, and were then dried. The urea-inclusion compound was then prepared by warming 3 kg of granular urea and 1 kg of corn oil ethyl esters with enough methanol to make hand stirring effective near the boiling point of methanol. Most of the methanol was then evaporated, and the inclusion compound was washed twice with light petroleum ether to remove any unbound esters.

Individual rats were inspected periodically for symptoms of essential fatty acid deficiency. Severity of symptoms was judged by scaliness of feet (range 0 to 3), scaliness of tail (range 0 to 4) and roughness of haircoat (range 0 to 2). Total scores were averaged for each group.

At the end of the experiment 4 of the 8 rats of each group were selected at random and killed. Each carcass was put through a meat grinder and then minced in a Waring Blendor with hot ethanol. The extraction was repeated twice with boiling ethanol and once with boiling Skellysolve F. The solvent was partially removed from the combined extracts,

the extract diluted with Skellysolve F, and washed three times with water. The lipid solution was then dried over anhydrous sodium sulfate, the solvent was removed and the lipid was weighed.

The content of arachidonic acid in the lipid samples was determined by alkaline isomerization using the conditions of Herb et al. ('52). Calculation of arachidonic acid content was made using the following equation, which provides appropriate corrections for the presence of pentaene and hexaene fatty acids:

$$\% \text{ arachidonic acid} = 100\,(0.165\,k_{3150\text{ Å}} - 0.0236\,k_{3460\text{ Å}} + 0.0079\,k_{3750\text{ Å}}).$$

$$k = \frac{\log I_o/I}{cd} \qquad \text{where c is measured in grams per liter.}$$

From the values so gained, and from the weight of the total lipid extracted, the total arachidonic acid per rat was calculated.

RESULTS AND DISCUSSION

The record of dermal symptoms during the course of the experiment is shown in figure 1. It will be noticed that those groups receiving the essential fatty acid, linoleic acid, either in corn oil or as the urea-inclusion compound of its ethyl ester, demonstrated parallel cures. Those groups of animals receiving no essential fatty acid showed roughly parallel changes in severity of symptoms. The animal colony is housed in a room without humidity control, and during the early summer the high humidity was reflected in spontaneous relief of dermatitis. However, later in the summer, the dermatitis of the fat-deficient groups again became severe, and the differences in responses of the various groups became more striking. The experiment demonstrated that from the point of view of response of dermal symptoms, essential fatty ester in urea-inclusion-compound form was effective in relieving fat deficiency.

Assessing the severity of essential fatty acid-deficiency by arachidonic acid content of the animals showed conclusively that essential fatty acid-deficiency was cured by corn oil

and by the urea-inclusion compound of corn oil esters. These results are shown in figure 2.

The use of high levels of urea in diets such as used here may exert some physiological strain upon the animals. Urea is a diuretic, and this was reflected in a higher water intake

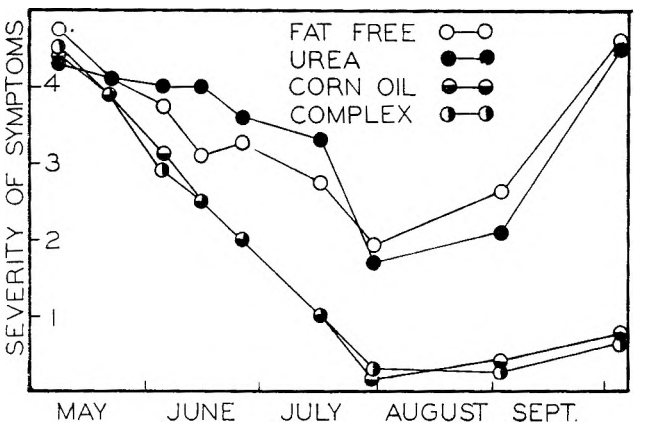

Fig. 1 Changes in dermal symptoms in fat-deficient rats supplemented with urea, corn oil or urea-inclusion compound (complex) of corn oil ethyl esters. Eight rats per group.

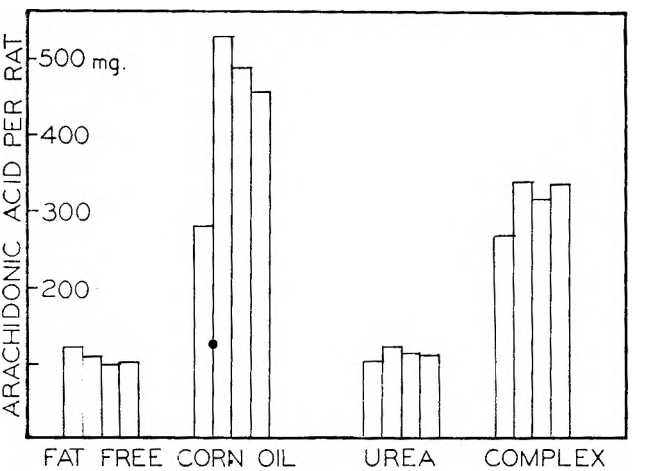

Fig. 2 Arachidonic acid contents of fat-deficient rats which had received supplements of corn oil, urea or urea-inclusion compound (complex) of corn oil ethyl esters. Each bar represents one rat.

of the animals receiving urea. The level of 6% urea in the diet lies within the therapeutic dose range of urea, and was not associated with any injurious effects upon the rats, although its dosage was continued over a period of several weeks.

Vitamin A or carotene present in an oil, or in a ration containing oil, is subject to coupled oxidation if the fatty portion of the ration undergoes autoxidation. It is conceivable that incorporation of unsaturated esters in a dry diet in the form of urea-inclusion compounds rather than as the free ester should lessen the oxidative destruction of these substances. To test this, two rations were compounded

TABLE 1

Stability of vitamin A in a dry diet in the presence of an urea-inclusion compound

RATION	RELATIVE OPTICAL DENSITY AT 3250 Å		VITAMIN A REMAINING
	Fresh	*40 days*	%
Vitamin A + ethyl linoleate	1.175	0.168	14.3
Vitamin A + ethyl linoleate urea-inclusion compound	1.15	0.745	65.0

similar to those described above. One contained 0.625 mg vitamin A plus 2 gm ethyl linoleate/100 gm ration. The other contained 0.625 mg vitamin A plus 8.0 gm ethyl linoleate urea-inclusion compound/100 gm ration. These rations were analyzed for their relative vitamin A content immediately and again after standing exposed to air at room temperature for 40 days. The unsaponifiable matter from 10 gm of each ration was taken up in 10 ml ethanol and the optical densities at 3250 Å were measured to indicate the relative amounts of vitamin A remaining (AOAC, '45). The data gained are summarized in table 1 and indicate that the loss of vitamin A was less when linoleate-inclusion compound was incorporated in the diet than when an equivalent amount of free ethyl linolete was present.

It is not recommended that urea-inclusion compounds be used routinely for the incorporation of fats in diets, but their use is desirable when supplementation with unsaturated fatty acids or esters is an essential part of an experiment. It is likely also that this method of supplementation can find use whenever an unstable straight chain aliphatic compound must be administered. For example, the continued administration of ethyl esters of tung oil fatty acids via their urea-inclusion compounds has been successfully practiced in our laboratory to avoid the oxidative polymerization of the eleostearic acid present in that oil.

SUMMARY

1. Comparison was made between the utilization of essential fatty acid (linoleic acid) in corn oil and the urea-inclusion compound of its ethyl ester. Changes in dermal symptoms and synthesis of arachidonic acid indicated that the essential fatty ester was active when administered in the form of its inclusion compound.

2. Vitamin A content of rations was found to decrease less over a period of 40 days if the essential fatty ester in the diet is present as urea-inclusion compound.

3. The use of urea-inclusion compounds of essential fatty acids is recommended as a means of supplementation by way of the ration, thereby affording protection of these unstable substances against autoxidative rancidity, and providing steady dosage.

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