HEPATIC NECROSIS INDUCED BY DIETARY MEANS

VIII. THE EFFECT OF FEEDING A DIET FREE FROM FAT AT SUCCESSIVE INTERVALS TO GROUPS OF WEANLING RATS

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The fact that the feeding of diets low in methionine, cystine and in alpha tocopherol causes acute massive liver necrosis in rats has been well established by a number of investigators (Gyorgy and Goldblatt, '39, '49; Dalt, Sebrell and Lillie, '42; Himsworth and Glynn, '44; Schwarz, '44a, b; Himsworth and Lindan, '49; and Abell and Beveridge, '49, '51). Although Himsworth et al. and Gyorgy et al. reported using diets low or high in fat no extensive investigation was made into the effects of the three main dietary components — protein, fat and carbohydrate — until the present authors performed such a study (McLean and Beveridge, '52).

The most striking results were obtained by varying the lard content of the diet from zero to 70%. All animals fed rations containing from 5 to 70% lard died of hepatic necrosis, the time required to produce the lesion increasing with decreasing fat content. All animals fed a diet free from fat survived the experimental period of 172 days. Microscopic examination of the livers of these animals revealed no evidence of liver necrosis. The fact that a diet free from fat prevented the development of acute liver damage was also reported simultaneously by Dam and Granados ('51) who, however, terminated their experimental period after an interval of only 70 days, much too short an interval to justify the conclusion reached by these workers. Furthermore, the incidence of liver necrosis in the group given the basal diet containing fat was

only 50%. In any event it appeared obvious that some constituent of the fat moiety of the diet either potentiated or was necessary for the production of the acute liver damage. During the course of further investigations designed to assess the necrogenicity of various types of lipids and lipid fractions a control diet free from fat was fed at successive intervals with results that are noted below.

TABLE 1

Composition of test diet

CONSTITUENTS	AMOUNT
	%
Primary grown yeast (Saccharomyces cerevisiae)[1]	18
Sucrose	75.98
Salts	3
Sugar vitamin mixture[2]	1
Celluflour	2
Cod liver oil concentrate (Ayerst, McKenna and Harrison; 50,000 I.U. vitamin D and 200,000 I.U. vitamin A per gram)	0.02

[1] Labeled Brewer's yeast U.S.P. by Nutritional Biochemicals Co., extracted three times with hot 95% alcohol.

[2] McLean and Beveridge, '53.

EXPERIMENTAL PROCEDURE

Male weanling rats of the Sprague-Dawley strain, average initial weight of about 50 gm, were housed in individual cages with one-third inch mesh screen floors and were offered daily approximately 8 gm of the test diet for periods of between 104–126 days. The composition of the diet is shown in table 1. The animals used in each experiment were obtained in separate shipments just prior to the commencement of the experiment. After each feeding trial the cages were disinfected and thoroughly washed with water. All studies were made in the same animal quarters maintained at a temperature of 68°–72°F. excepting during the summer months when the temperature usually ranged between 68°–80°F. The experiments were carried out over a period of 15 months. The diagnosis of liver necrosis was based always on a microscopic examination of

sections made from the three main lobes of the liver, stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

The results obtained are shown in table 2. The absence of fat in the diet initially protected the animals from the development of acute hepatic damage. However, when the same diet was utilized in three subsequent experiments an increasingly

TABLE 2

The effect of feeding a diet free from fat at successive intervals to groups of rats

EXPERIMENT NO.	DURATION OF EXPERIMENT	INCIDENCE OF NECROSIS		TIME AT WHICH DEATH OCCURRED DUE TO LIVER NECROSIS			
		Fat-free diet	Fat-containing diet [2]	Fat-free diet		Fat-containing diet	
				Average	Range	Average	Range
	days			*days*	*days*	*days*	*days*
1	172	0/5	5/5	. .	. . .	73 (52)[4]	38–157
2	104	1/10	9/10	50	. . .	40	28–104
3	126	3/10	. .[3]	99	77–119	. .	. . .
4	105	8/9[1]	10/10	56	49–92	39	23–70
5	105	4/10	8/10	65	44–79	49	30–78

[1] One animal died at 35 days of causes other than liver necrosis.

[2] These diets contained 5% lard with the exception of the diet used in experiment two in which 7% lard was used.

[3] No data are available since a diet containing lard was not fed at this time.

[4] One animal died of liver necrosis 91 days after the remaining 4 animals had all succumbed; if this animal is excluded from the calculation a more representative average of 52 days is obtained.

high incidence of hepatic necrosis occurred. A somewhat lower incidence of necrosis was obtained in the last experiment.

The appearance of massive liver necrosis in the control animals receiving a ration free from fat was most unexpected and since there was a general trend towards an increasing incidence of the lesion this circumstance made practically impossible a valid assessment of the relative necrogenic qualities of the different lipids and lipid fractions that were being tested. For this reason the data on the necrogenicity of the various lipids are not being reported at this time pending further investiga-

tion. However, in view of the fact that in 4 separate well controlled experiments acute liver necrosis was found to develop on fat-free diets, it was believed worthwhile to publish these data in order to make it clear that these rations do not necessarily completely prevent the appearance of the hepatic lesion, as might be inferred from an examination of the papers by Dam and Granados ('51) and ourselves (McLean and Beveridge, '52).

The possibility that seasonal variations might be responsible for the results has been considered and discarded on the basis that the experiments covered a period of about 15 months and for the additional reason that during many previous experiments carried out in this field during 6 years no effect of season has ever been observed on the development of liver necrosis. Gyorgy, Stokes and Goldblatt ('51) were also unable to show that seasonal variation played a role in the production of this lesion.

It may be of interest to note that in the type of liver injury produced by feeding alkali-treated casein, Schwarz ('44a) reported a variation in the incidence of liver damage that he ascribed to seasonal variation. Apart from the fact that the etiology of the lesion described by this investigator differs from the one reported in the present paper, Schwarz' data can hardly be taken as indicative of a consistent effect due to seasonal variation. In this same paper, data were published demonstrating that the average time of development of acute liver damage was essentially the same on a diet free from fat or on one containing 10 or 20% butter fat.

Although Naftalin ('51) has shown that of the three ranges of environmental temperatures studied, 60°–64°F., 70°–78°F., and 88°–92°F., the best temperature for the production of acute liver necrosis was 70°–78°F., this work has little significance in the present instance since the animal room was maintained at a temperature of 68°–72°F. excepting during the summer months when the temperature usually ranged between 68°–80°F.

The possibilities that the changing of dietary ingredients or changing susceptibility of the animals, or both, have been also considered and discarded as explanations for the results due to the fact that there was no progressive increase in susceptibility to the development of hepatic damage in the animals receiving diets containing fat. This point is best illustrated by a comparison of the results obtained in experiments 2 and 4 in table 2. The incidences of necrosis on the fat-containing diets and the average times at which death occurred due to the acute liver lesion were essentially identical. On the other hand, the corresponding data for the fat-free diets differed considerably, being 1/10 and 50 days in experiment two, and 8/9 and 56 days in experiment 4.

In an attempt to present a rational explanation for the results reported here we should like to call attention to the work of Gyorgy et al. (Gyorgy, Stokes, Smith and Goldblatt, '50; Gyorgy, Stokes, Goldblatt and Popper, '51) on the ameliorative effect of certain antibiotics on the development of dietary hepatic necrosis.

It is of interest to note that Gyorgy, Stokes and Goldblatt ('51) found that when aureomycin was used in successive experiments it gradually lost its anti-necrogenic property — a situation that is somewhat analogous to our own experience in utilizing diets free from fat at successive intervals. Gyorgy, Stokes, Goldblatt and Popper ('51) have suggested that the anti-necrogenic action of aureomycin is due to its "suppression of the intestinal flora, or at least of some of its constituents, and thus prevents the formation of bacterial metabolites with which the liver, in the absence of vitamin E or of sulfur-containing amino acids as detoxifying agents, is unable to cope." The progressive decrease in the delaying effect of aureomycin noted in successive experiments led them to postulate "that in the course of one and one-half years either aureomycin resistant strains of intestinal bacteria developed in the animal room and were transmitted from one experiment to the following one or that the virulence of some unrecognized infectious factor, such as a virus, increased to such an extent

that the aureomycin effect was obscured." The former sequence of events might well be responsible for the results obtained with the feeding of the diet free from fat to successive groups of rats. In any event, regardless of the validity of the foregoing hypothesis, there can be no doubt that the nature of the intestinal flora directly or indirectly plays an important role in determining the susceptibility of rats to the development of dietary liver necrosis. This statement is firmly supported by the work reported by Gyorgy and his colleagues on the effect of various antibiotics and on germ-free experiments carried out in collaboration with Reyniers and his associates at the Germ-free Life Laboratory, University of Notre Dame — (Gyorgy, Stokes, Smith and Goldblatt, '50; Gyorgy, Stokes, Goldblatt and Popper, '51; Gyorgy, Stokes and Goldblatt, '51). In the latter work the germ-free rats lived twice as long as the control rats outside the germ-free laboratory, and at autopsy no necrosis of the liver was found. Experiments by Abell and Beveridge ('51) have also shown that both sulphaguanidine and aureomycin prolong the length of time required to produce acute hepatic damage in rats.

The suggestion that such a drastic dietary alteration as rendering the diet free from fat would cause marked alterations in the intestinal flora would appear to be justified since it has been reported by several investigators that dietary alterations are reflected by changes in the intestinal flora (Kendall, '09; Rettger, '15). If it is assumed that the feeding of the fat-free diet changed the bacterial flora in the animals, the repetition of the dietary regime might be considered to be responsible for the production of a number of sub-cultures. It has been demonstrated by *in vitro* work that under such circumstances some bacteria may adapt themselves so as to achieve maximal growth in the altered nutritional environment (Silverman and Werkmann, '39).

Although the authors could find in the literature no direct reference to the transmission of intestinal bacteria where the animals did not have access to each others' feces, that this type of transfer can take place is evident from the tenacity

with which such intestinal infections as salmonella remain in animal quarters.

SUMMARY

A diet free from fat was fed to 5 successive groups of male weanling rats over a period of 15 months.

In the first experiment none of the rats devleoped hepatic necrosis in a period of 172 days. In all subsequent groups hepatic necrosis developed with a general trend towards an increasing incidence.

It is tentatively suggested that these results might be explained on the basis that intestinal organisms having a strong predisposing influence on the production of the lesion were inhibited in the first case and subsequently became adapted to the altered dietary conditions with the consequent production of liver necrosis.

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NUTRITIONAL STUDIES WITH THE GUINEA PIG

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TWO FIGURES

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No account of an uncomplicated pantothenic acid deficiency in the guinea pig has been made, so far as the authors are aware, other than a preliminary report by Reid ('52). Since suggestions have been advanced that a functional interrelationship exists between ascorbic acid and pantothenic acid, it seems important to study the possibility of such an interdependence with respect to these substances in the guinea pig, an animal which requires a dietary source of ascorbic acid. For these reasons we wish to present additional information on pantothenic acid studies with this animal including a description of the gross deficiency symptoms, a determination of the approximate requirement by very young animals and the relation of the ascorbic acid content of the diet to the pantothenic acid requirement.

EXPERIMENTAL PROCEDURE

Guinea pigs of the Hartley strain, ranging in age in different experiments from one to 21 days and from 91 to 295 gm in weight, were placed in wire screen-bottomed cages and for a 6-week period were fed a "synthetic" diet previously described (Reid and Briggs, '53) containing 30% vitamin-free casein, 7.3% fat, 4 types of carbohydrates, 6% salts and liberal amounts of the known vitamins except panto-

thenic and ascorbic acids as indicated. Care of the animals was according to procedures previously described (Reid and Briggs, '53).

RESULTS

Two- to 4-day-old animals fed the pantothenic acid-deficient diet containing 0.2% ascorbic acid grew poorly and had a high mortality rate. Figure 1 shows the characteristic appearance of the deficient and normal control animals on the 25th day of the experiment. The chief gross symptoms of pantothenic acid deficiency were a decrease in rate of growth, followed by a loss of weight, roughening of the coat, tendency

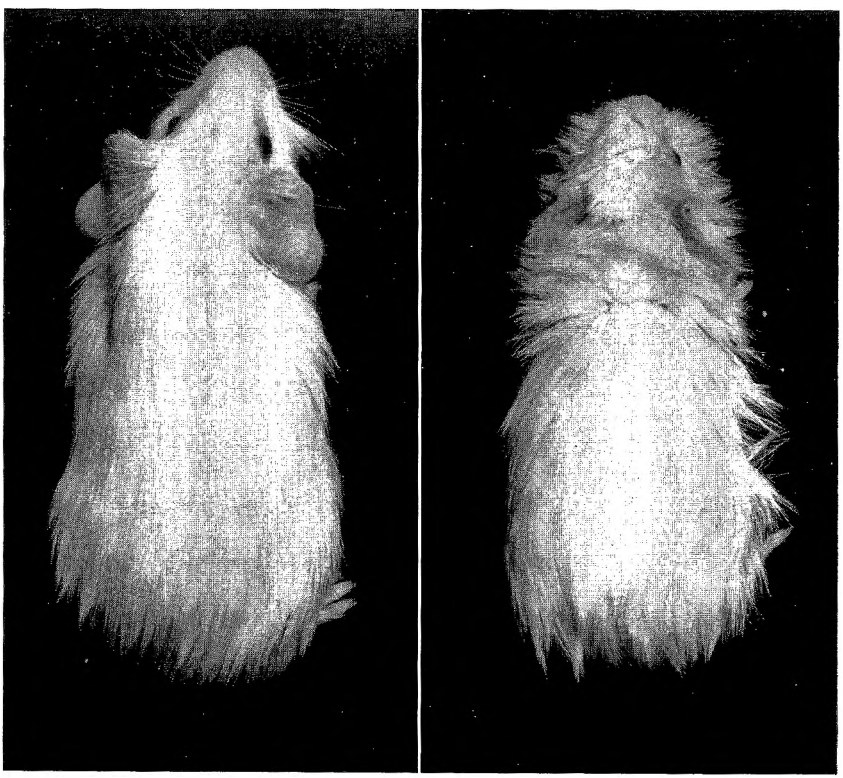

Figure 1

Left: Normal animal after 25 days on complete diet.
Right: Animal after 25 days on pantothenic acid-deficient diet.

to diarrhea, anorexia, weakness, inactivity and death. There was no evidence of porphyrin-staining of the fur and whiskers and no "spectacled-eyes." In animals with black colored areas (Beltsville strain), reared on a diet lacking pantothenic acid, there appeared to be some dulling of the colors but no clear evidence of loss of pigmentation. In further tests with a few animals in which portions of the black areas of the fur were shaved, the new hair which grew in showed no change of color. There was a marked difference between the control and deficient animals in fat deposition around the organs, particularly around the kidneys. However, in pair-fed controls, fat deposition appeared to be reduced almost as much as in the deficient animals. The adrenals of the deficient animals were considerably enlarged, some of them became hyperemic and in several of the animals which were found dead, the adrenals were hemorrhagic. The immediate cause of death was not ascertained.

AGE OF ANIMALS AND DEVELOPMENT OF DEFICIENCY SYMPTOMS

Like in most other animals, the rate of onset of the deficiency is related to the age and weight at which the animals are placed on the deficient diet. Figure 2 shows the results of an experiment with three groups of 8 animals each, one group on the normal complete diet and the other two groups on a diet lacking pantothenic acid. The deficient groups varied slightly in their starting weight and differed somewhat more in age. The lower curve in the figure represents the average weights, at successive periods, of animals placed on the diet at two to 4 days of age with an average weight of 101 gm and a weight range of 98 to 107 gm. The average time required for the attainment of maximum weight in this group was 13 days and the average survival time was 24 days. The next curve in the figure represents the results of a similar experiment with animals placed on the diet at 4 to 10 days of age with an average initial weight of 108 gm and a weight

range of 96 to 137 gm. On the 47th day on the diet, two animals were still living. The average survival time of the 6 animals which died was 30 days and the average time required for the individuals to reach their maximum weight was 22 days. The differences in survival time, percentage survival

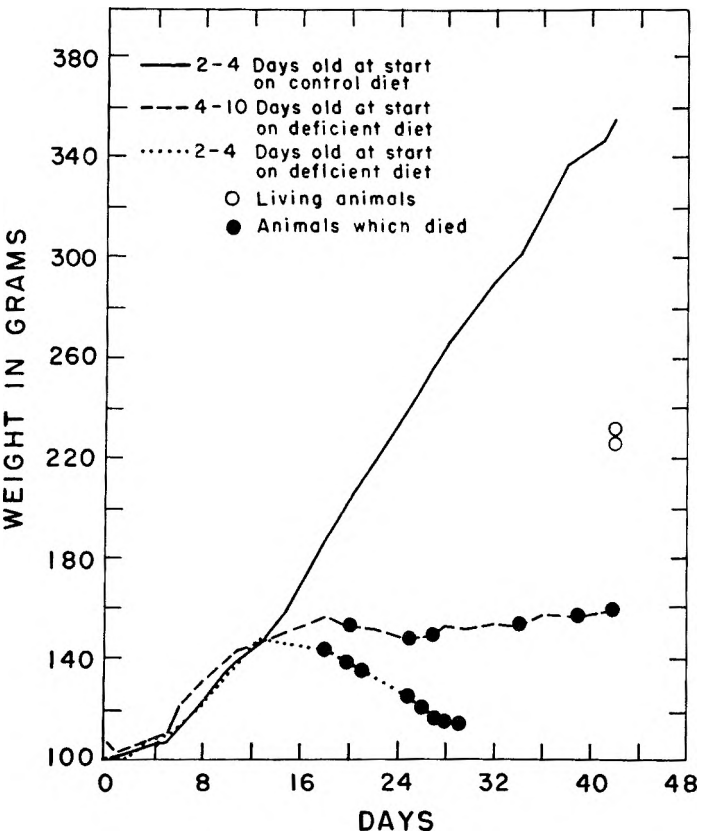

Fig. 2 Average weight curves of pantothenic acid-deficient guinea pigs and their controls (8 animals per group) started on the diets at different ages.

As animals died their final weight was used in calculating the average weight of the entire group on succeeding days of the experiment. A lop-sided picture of the effect of the deficiency results if the weights of only the surviving (heavier) animals are used. However, it is recognized that neither method of representing the growth curve is without objection if the mortality rate is high.

The two circles off the curve represent the average weight on the 42nd day of the two surviving animals in the group started on the deficient diet at 4 to 10 days of age.

and the average time required to attain maximum weight in the animals which succumbed to the deficiency, have been corroborated in the results of several other experiments. In the experiments started with the older animals there usually were a few individuals which appeared to have the capacity to survive more or less indefinitely, but all the others developed an unthrifty appearance and eventually died. The third curve in figure 2 shows the average rate of growth of the normal control animals receiving 40 mg calcium pantothenate per kg of diet.

REQUIREMENT FOR PANTOTHENIC ACID

Several experiments were conducted to determine the minimum requirement of calcium pantothenate in the diet. Levels of 10, 15, 20, 30 and 40 mg per kg of diet (containing 0.2% ascorbic acid) were studied using 8 animals per group. The results shown in table 1 are expressed in terms of the number of fatalities and the weights of survivors after 6 weeks on the respective diets. A level of 10 mg was not enough to permit maximum growth and survival. Whether the greater incidence of deaths at this level was due entirely to an inadequate amount of the vitamin or in part to greater susceptibility to infection (Ludovici et al., '49) was not determined. Essentially the same results were obtained with 20, 30 and 40 mg/kg. Fifteen mg may possibly be sufficient. A difference of one or two days in the age at which the guinea pigs were removed from the mother appeared to affect the requirement just as in the case of the previously mentioned growth and survival with complete absence of the vitamin from the diet. The animals used in the first experiment (table 1) were one to two days older than those used in the second and third experiments and a difference is to be noted in the growth at levels of 10, 15 and 20 mg. There were no fatalities in the first experiment at the 10 mg level whereas there were 5 in the second and three in the third experiment.

INTERRELATIONS OF ASCORBIC AND PANTOTHENIC ACIDS

Since it had been shown that the development of pantothenic acid deficiency symptoms in the rat could be largely prevented by the addition to the diet of 2% ascorbic acid (Daft, '51; Daft and Schwarz, '52) it seemed desirable to conduct investigations on possible dietary interrelationships of pantothenic and ascorbic acids in the guinea pig. Table 2 shows the results of one of these experiments in which 5 groups of 8 animals each were employed, all reared on a diet lacking pantothenic acid. In this experiment there was a wide initial weight range (82 to 144 gm) among the individuals of a group. The results show that animals of this particular experiment subjected to lack of both ascorbic and pantothenic acids in the diet succumbed earlier than those on pantothenic acid deficiency alone and also earlier than those on ascorbic acid deficiency.[1] No definite benefit appears to have been derived from the presence of large amounts of ascorbic acid in the diet of the guinea pig. In this respect the guinea pig differs markedly from the rat.

The problem of pantothenic and ascorbic acid interrelations was also studied from another approach, namely, the effect of the ascorbic acid content of the diet on the ability of guinea pigs to survive and grow on suboptimum, as compared to optimum, levels of pantothenic acid. A supply of 10 mg of calcium pantothenate per kg of diet was employed as the suboptimum amount and 20 mg per kg as the optimum. Levels of ascorbic acid varied from 0.1% to 1.0%, even the lowest amount being sufficient to protect the animals from scurvy. Results of the experiment are summarized in table 3. On the 20 mg level of calcium pantothenate there were no fatalities in any of the groups whereas at the 10 mg level there were fatalities in three of the 4 experimental groups with the highest incidence occurring at the 0.1% level of ascorbic acid. In another experiment, the results of which are not shown, conducted with slightly older animals, 10 mg per kg of calcium pantothenate was added to the diets for

[1] Unpublished data obtained in experiments with the same basal diet.

TABLE 1

Average weights (gm) with standard errors[1] *at 6 weeks of animals (8 per group)*[2] *reared on different levels of calcium pantothenate (All diets containing 0.2% ascorbic acid)*

EXP. NO.	RANGE OF AV. INITIAL WTS. PER GROUP	LEVELS OF CALCIUM PANTOTHENATE				
		10	15	20	30	40
	gm	*mg/kg*	*mg/kg*	*mg/kg*	*mg/kg*	*mg/kg*
1	106–110	310 ± 16	369 ± 14	375 ± 17	...	365 ± 21
2	98–102	211 ± 20 (5)	312 ± 18	325 ± 18	346 ± 12	...
3	100–101	310 ± 17 (3)	...	344 ± 21 (1)	345 ± 21	350 ± 18

[1] Determined by method of Mantel ('51).
[2] Figures in parentheses indicate the number of animals which died.

TABLE 2

Effect of ascorbic acid on growth and survival of guinea pigs reared on diets lacking pantothenic acid (8 animals per group)

ASCORBIC ACID IN DIET	AV. INITIAL WT.	AV. WTS. OF SURVIVORS AFTER SUCCESSIVE WEEKS ON DIETS[1]						AV. SURVIVAL TIME OF THOSE DEAD BEFORE 6 WKS.
		1	2	3	4	5	6	
%	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*days*
0.0	110 ± 5	122	146	137	123 (6)	... (8)	... (8)	25
0.1	111 ± 6	126	160	163	173 (2)	201 (4)	233 (5)	29
0.125	108 ± 3	124	157	149 (1)	176 (4)	212 (5)	251 (5)	26
0.5	110 ± 5	132	165	158	161 (4)	187 (7)	... (8)	29
2.0	110 ± 6	120	149	158	169 (2)	204 (4)	213 (4)	27

[1] Figures in parentheses indicate the number of animals which died.

two groups of 8 animals each; one group was on a 0.1% level of ascorbic acid and the other on 0.2%. There was 100% survival in both groups and no significant difference in the average final weights. The results suggest, but do not prove, that the age at which the animals are removed from the mother affects the response to the level of both pantothenic and ascorbic acids.

TABLE 3

Growth and survival of guinea pigs maintained on different levels of calcium pantothenate and ascorbic acid (8 animals per group)

PERCENTAGE CONTENT OF ASCORBIC ACID	NO. OF FATALITIES	INITIAL WT.	AV. WTS.[1] AFTER SUCCESSIVE WEEKS ON DIETS		
			2	4	6
			10 mg calcium pantothenate per kg		
		gm	*gm*	*gm*	*gm*
0.10	3	105 ± 2[2]	158 ± 6	185 ± 13	257 ± 22
0.25	2	105 ± 2	163 ± 7	226 ± 12	307 ± 20
0.50	0	105 ± 2	159 ± 8	210 ± 9	285 ± 7
1.00	1	106 ± 2	157 ± 7	218 ± 16	299 ± 24
			20 mg calcium pantothenate per kg		
0.10	0	105 ± 2	161 ± 6	250 ± 11	304 ± 22
0.25	0	105 ± 2	162 ± 8	244 ± 10	299 ± 18
0.50	0	106 ± 2	167 ± 7	245 ± 5	294 ± 9
1.00	0	103 ± 2	153 ± 7	238 ± 13	309 ± 19

[1] Of surviving animals.

[2] Standard error.

In preliminary studies of the blood of animals started on the diet at two to 4 days of age with calcium pantothenate in the diet at levels of 0, 10 and 40 mg, no significant differences were found in the hematocrit, hemoglobin, total leukocytes and granulocytes. The blood samples were taken when the animals on the diet lacking pantothenate had plateaued in weight but before they had shown signs of severe deficiency.

DISCUSSION

It is not surprising that the guinea pig requires a dietary source of pantothenic acid since a dietary supply of this

vitamin is known to be required by almost all vertebrates studied so far, including the rat, dog, mouse, monkey, chicken, turkey, duck, pigeon, pig, calf, fish, fox, hamster and possibly man (Robinson, '51). The deficiency symptoms described herein for the guinea pig are similar, in general, to those commonly seen in several other animals.

The precise requirement of pantothenic acid cannot be decided from the data but it appears to be between 15 and 20 mg per kg of diet for very young animals. This is somewhat higher than the requirement reported for most other animals (Robinson, '51) and is possibly a consequence of the use in these studies of animals which had suckled for a much shorter period than had the different types of animals employed by other investigators.

The inability to obtain achromotrichia in the guinea pig as a consequence of pantothenic acid deprivation is somewhat surprising compared with the apparent ease of obtaining this condition in black rats and mice (Robinson, '51) reared on diets lacking this vitamin. Morgan and Sims ('40) reported that one guinea pig out of 4 developed gray hair after two months on a diet consisting of casein, sucrose or cornstarch, agar, salts, Crisco, extracted wheat germ and with daily supplements of ascorbic acid, nicotinic acid and cod liver oil. This animal died after two weeks' treatment with a crude preparation designated as "anti-grey factor." The diet was undoubtedly deficient in certain B-vitamins, especially folic acid (known to prevent achromotrichia in chicks [Frost, Dann and McIntire, '46; Lillie and Briggs, '47]) in addition to the filtrate factor. No weight values are given and it must be concluded that the results are too unconvincing and obtained with too few animals to justify attributing them to lack of pantothenic acid. Achromotrichia in the guinea pig due to uncomplicated lack of pantothenic acid has, therefore, not yet been produced.

The sparing effect of large amounts of dietary ascorbic acid on pantothenic acid deficiency in the rat (Daft, '51; Daft and Schwarz, '52) could not be duplicated in the guinea

pig. Further studies are required, however, before drawing the conclusion that in diets containing suboptimum amounts of pantothenic acid no benefit is derived from the presence of dietary ascorbic acid in excess of the amount necessary to prevent scurvy.

The reason for a smaller requirement of pantothenic acid by the older animals may be a consequence of (1) greater storage of pantothenic acid (2) greater inoculation of the gastrointestinal tracts with beneficial microorganisms, some of which may have the capacity to synthesize pantothenic acid and (3) greater transfer of immune bodies (Maynard, '51) in the animals which suckled longer. Presumably these factors may all exert an influence. Removal of the young from the mother within two to 4 days after their birth would obviously affect all of these factors. The difference in the requirement of pantothenic acid by different types of young animals may therefore possibly be a consequence not of differences in age as such (since the guinea pig has a gestation period of 70 days as compared to the rat in which it is 21 days) but may rather be a consequence of differences in the length of time during which the young remain with the mother following their birth.

SUMMARY

Young guinea pigs, supplied with a "synthetic" diet lacking pantothenic acid, developed deficiency symptoms characterized by a decrease in the rate of growth, followed by a loss of weight, roughening of the coat, a tendency to diarrhea, anorexia, weakness, inactivity and eventual death. The internal symptoms were a decrease in fat deposition, with hyperemia, enlargement, and, in some cases, hemorrhages of the adrenals. The rate of onset of the deficiency was found to be related to the age and weight at which the animals were placed on the diet. Preliminary studies indicate no clear evidence of a disturbance of the blood picture in the early and mid-stage of the deficiency.

The young guinea pig's requirement of pantothenic acid appears to be between 15 and 20 mg per kg of diet. In older

animals the requirement appears to be less. Unlike the rat, no benefit appeared to be derived from the presence of large amounts of ascorbic acid in the deficient diet.

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PREVENTION OF THE CHOLESTEROL TYPE OF FATTY LIVERS IN MICE BY DIETARY DIHYDROCHOLESTEROL

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The relationship of the diet to cholesterol absorption, distribution and balance has been the subject of extensive investigation for many years. Recently a number of papers have appeared indicating the usefulness of soybean sitosterols (Sperry and Bergmann, '37; Peterson, '51; Peterson, Nichols and Shneour, '52; Pollak, '53; Peterson, Shneour, Peek and Gaffey, '53) and dihydrocholesterol (Siperstein, Nichols and Chaikoff, '53) in the control of cholesterol absorption. These efforts so far have been chiefly concerned with the prevention of cholesterol-induced atherosclerosis in chicks and rabbits. Efforts to lower organ and blood cholesterol in mice by means of soybean sitosterols have been unsuccessful (Schettler, '48). Our present experiments have been designed to determine whether the cholesterol type of fatty livers in mice can be prevented by dihydrocholesterol, and to gain some knowledge about the mechanism of this action.

EXPERIMENTAL

Webster strain male albino mice weighing 18 to 20 gm were used. After an observation period of two weeks, the mice were weighed, housed in separate cages and divided into 7 groups, each with 7 to 9 animals. Each group was fed a basal cholesterol-free diet supplemented as listed in table 1 with cholesterol, cholic acid and dihydrocholesterol.[1]

[1] Generously supplied by the Schering Corporation, Bloomfield, New Jersey.

The basal cholesterol-free diet used in all of the experiments had the following composition: vitamin-free casein 25, sucrose 55, non-nutritive diet 16 (alphacel)[2], and salt mixture 4% (Phillips and Hart, '35). This diet was supplemented with the following quantities of vitamins, expressed as milligrams per kilogram: thiamine hydrochloride 40.4, riboflavin 60.8, calcium pantothenate 151, niacin 1,008, pyridoxine hydrochloride 40.4, 2-methyl-1,4-naphthoquinone 5.12, alpha-tocoperol 30.2, folic acid 20.3, biotin 2.02, choline chloride 20,200, *p*-aminobenzoic acid 202, inositol 10,100 and ascorbic acid 100.

After an experimental period of three weeks, all mice were weighed and sacrificed by decapitation. The livers were rapidly removed, weighed and analyzed for total cholesterol and total β steroids as follows:

The excised liver was placed in a round-bottom flask containing 20 ml of 70% ethyl alcohol and 1.5 gm of potassium hydroxide. The mixture was refluxed on a steam bath for three hours. The resulting clear brown liquid was adjusted to pH 7 and transferred to a 50-ml volumetric flask using 95% alcohol. A 20-ml aliquot was placed in a separatory funnel, 40 ml of water added, and the mixture extracted with four 20-ml portions of ether. The ether extracts were evaporated to dryness and then transferred to a 25-ml volumetric flask by means of a 1:1 ethyl alcohol-acetone mixture. Cholesterol was determined in aliquots of this solution by the Schoenheimer-Sperry method as modified by Sperry and Webb ('50).

Total β steroids were determined by utilizing the well-known reaction of digitonin and anthrone. The technique, as developed in this laboratory, is as follows: The digitonin precipitate was prepared and washed as in the Schoenheimer-Sperry method, except that an additional washing with 2 ml of 50% ethyl alcohol was found necessary before washing with acetone ether. The washed precipitate was dissolved in glacial acetic acid and diluted with glacial acetic acid to 10 ml. A 2-ml aliquot

[2] Purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

was placed in a cuvette and 3 ml of a 2% solution of anthrone in glacial acetic acid were added. At one-minute intervals, 5 ml of concentrated sulfuric acid were added to successive tubes as rapidly as possible with vigorous agitation. The tubes were placed in the dark at room temperature for 30 minutes and then read in the spectrophotometer at 670 mμ. Blanks and standards were prepared by using 2 ml of glacial acetic acid and 2 ml of digitonin in glacial acetic acid (0.1 mg %) in place of the unknown. This method, which is about 10 times as sensitive as the Lieberman-Burchard reaction, is useful for the determination of digitonin-precipitable steroids and especially for dihydrocholesterol, which does not yield a color under the conditions of the Lieberman-Burchard reaction.

RESULTS

The results presented in table 1 show that the addition of cholesterol and cholic acid to the cholesterol-free diet (I) increases total liver cholesterol and total β steroids (diets II, III and IV). When the cholic acid of the diet is doubled (diet III) or quadrupled (diet IV), total liver cholesterol and β steroids increase, but reach a limiting value (diets III and IV). The addition of dihydrocholesterol to diets containing cholic acid and cholesterol (diets V, VI and VII) results in a significant decrease in total liver cholesterol and total β steroids. If cholesterol and dihydrocholesterol concentrations are held constant, and cholic acid concentration is increased stepwise, as in diets V, VI and VII, total liver cholesterol and β steroids decrease significantly as cholic acid concentration rises. Total liver cholesterol and total β steroids in group VII were significantly lower than in the control group (group I). Comparison of the results obtained using the anthrone method and the Lieberman-Burchard method shows that, under our experimental conditions, no dihydrocholesterol is deposited in the liver. In all cases, total β steroid concentration parallels total cholesterol concentration.

TABLE 1

Effect of dietary dihydrocholesterol and cholic acid on total liver cholesterol and β steroids

GROUP AND DIET	NO. OF MICE	ADDITIONS TO CHOLESTEROL-FREE DIET			TOTAL CHOLESTEROL IN LIVER		TOTAL β STEROIDS IN LIVER	
		Cholesterol	Cholic acid	Dihydro-cholesterol	Per organ [2]	Per gram body weight	Per organ [2]	Per gram body weight
		%	%	%	*mg*	*mg*	*mg*	*mg*
I	9	..	..	..	9.7 ± 1.6 [1]	0.39 ± 0.07 [1]	12.2 ± 3.0 [1]	0.48 ± 0.18 [1]
II	9	1.0	0.25	..	21.4 ± 6.3	0.85 ± 0.14	22.2 ± 6.2	0.96 ± 0.23
III	7	1.0	0.5	..	30.2 ± 5.5	1.50 ± 0.36	33.2 ± 5.0	1.6 ± 0.22
IV	7	1.0	1.0	..	32.1 ± 5.3	1.50 ± 0.30	32.0 ± 5.4	1.5 ± 0.40
V	8	1.0	0.25	2.5	11.4 ± 4.5	0.51 ± 0.15	13.3 ± 5.5	0.79 ± 0.32
VI	9	1.0	0.5	2.5	7.6 ± 3.1	0.35 ± 0.11	9.3 ± 4.3	0.44 ± 0.18
VII	7	1.0	1.0	2.5	5.1 ± 0.8	0.28 ± 0.04	6.8 ± 1.1	0.35 ± 0.18

[1] Standard deviations.

[2] Significance of the difference in results of the following groups: (a) II and III, $P < 0.01$; (b) II and V, $P < 0.01$; (c) V and VI, $P \cong 0.05$; (d) V and VII, $P < 0.01$; (e) VI and VII, $P \cong 0.05$.

DISCUSSION

It is clear from the data shown above that under our dietary conditions the concentrations of dihydrocholesterol employed were very effective in preventing the cholesterol type of fatty liver in mice. In an experiment reported by Hernandez et al. ('53), it has been shown that soybean sitosterols inhibit the absorption of dietary cholesterol. It is reasonable to assume that dihydrocholesterol acts in a similar manner. At least three possible mechanisms are suggested: (1) some type of nonabsorbable complex is formed; (2) dihydrocholesterol combines with all the available bile salts, forming stable complexes or emulsions, so that insufficient bile salts remain for the absorption of cholesterol; or (3) dihydrocholesterol inhibits intestinal cholesterol esterase or other enzymes necessary for cholesterol absorption. The second possibility has been eliminated by the present experiment, inasmuch as an increase in bile acid, in the presence of cholesterol and dihydrocholesterol, decreased rather than increased liver cholesterol. The other possibilities are being investigated.

SUMMARY

1. Increasing the cholic acid content of a diet containing cholesterol significantly increased mouse liver cholesterol and β steroids, up to a limiting value.

2. The addition of dihydrocholesterol to diets containing cholic acid and cholesterol significantly decreased mouse liver cholesterol and β steroids.

3. Increasing the cholic acid content of a diet containing constant amounts of dihydrocholesterol and cholesterol significantly decreased mouse liver cholesterol and β steroids.

4. Under all conditions, total liver cholesterol and β steroid concentrations were parallel.

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COMPARISON OF FRUCTOSE AND GLUCOSE IN THE DIET OF ALLOXAN-DIABETIC RATS [1]

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THREE FIGURES

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Recent studies have shown that fructose is rapidly converted to fructose-1-phosphate in liver and muscle and enters the glycolytic cycle by a different pathway than that used for glucose (Vestling et al., '50; Cori et al., '51; Hers, '52). This phosphorylation of fructose is not affected by insulin or glucose (Cori et al., '51; Mackler and Guest, '53). Liver slices of alloxan-diabetic rats oxidize fructose at a normal rate and glucose at a depressed rate (Chernick and Chaikoff, '51). Also the utilization of intravenously administered fructose is normal in pancreatectomized dogs (Pletscher and Hess, '51) and in diabetic human subjects (Craig et al., '51; Miller et al., '52b). Because fructose has this different metabolic pathway it is of interest to note that recent clinical reports have shown fructose to have definite advantages over glucose when given intravenously to subjects with diabetes mellitus (Dolger et al., '53; Darragh et al., '53).

In the present study, experiments were carried out using alloxan-diabetic rats to determine whether a diet containing fructose as the only carbohydrate would be utilized differently from a diet containing glucose. The data indicate that the degree of glycosuria and polyuria is significantly less in

[1] Presented before the Society for Pediatric Research, Atlantic City, New Jersey, May 4–6, 1953.

alloxan-diabetic rats fed the fructose diet than in those fed the glucose diet.

EXPERIMENTAL

Male weanling rats were placed on an 18% casein diet containing dextrinized corn starch (table 1) for 4 weeks, fasted for 72 hours, and injected intravenously with a fresh 5% solution of alloxan monohydrate at a level of 40 mg per

TABLE 1

Composition of experimental diets

	gm per 100 gm
Casein, Labco vitamin free	18
Corn oil, Mazola	10
Carbohydrate [1]	64
Salt mixture [2]	4
Non-nutritive fiber (General Biochemicals)	3.65
Vitamin mixture [3]	0.35

[1] Dextrinized corn starch (Amidex), fructose or glucose.

[2] Jones and Foster ('42) salt mixture with NaF added to give 10 p.p.m. F in salt mixture.

[3] Vitamin mixture, per 100 gm of diet.

Thiamine hydrochloride	0.4 mg	Folic acid	0.20 mg
Riboflavin	0.5 mg	Biotin	0.02 mg
Niacinamide	5.0 mg	Vitamin B_{12}	0.01 mg
Pyridoxine hydrochloride	0.25 mg	Menadione	0.2 mg
Calcium pantothenate	2.0 mg	Ascorbic acid	20.0 mg
Choline bitartrate	200.0 mg	α-tocopherol	5.0 mg
Inositol	100.0 mg	Oleum Percomorphum	0.015 ml
p-Aminobenzoic acid	10.0 mg		

kilogram. (Some of the animals were injected with saline solution and kept as controls.) The animals were continued on the dextrin diet and blood sugar levels were measured 48 hours later and at weekly intervals thereafter by the method of Nelson ('44). In the first metabolic studies 7 rats were transferred to the glucose diet and 7 to the fructose diet, three weeks after the animals had been made diabetic. Some of the animals recovered spontaneously on these diets leaving 4 diabetic animals on the fructose diet and 6 on the glucose diet. During the course of this experiment the diets of

the animals were changed from fructose to glucose and from glucose to fructose in order to allow each animal to serve as its own control. In the second experiment, the rats were kept on the dextrin diet for three months after alloxan injection in order to minimize the chance of spontaneous recovery later in the experiment. Two groups of 10 rats each were selected on the basis of water intake and weekly blood sugar values, placed in individual metabolism cages, and continued on the dextrin diet for 12 days before changing to the fructose and glucose diets.

The level of total blood sugar was measured each week and records were kept of food and water intake. Urine was collected daily and pooled in two or three day samples for analysis of total reducing sugar (Nelson, '44). Urinary fructose was also measured in animals on the fructose diet, using a modification (Weichselbaum, '52) of the method of Roe ('34) but blood fructose levels were not determined. At the conclusion of each experiment the animals were fasted for 24 hours and sacrificed by nembutal anesthesia. The livers were rapidly removed, frozen in liquid nitrogen, weighed, and digested in 40% KOH solution. The liver glycogen was precipitated with alcohol and measured by a modification of the anthrone method (Scott and Melvin, '53). The kidneys and adrenal glands were weighed and fixed for histologic study. The carcasses were weighed in tared, covered pint Mason jars, autoclaved at 15 pounds' pressure for 4 hours and saved for analysis of solids, total lipid, protein and ash. Each carcass was homogenized in the Waring blendor and samples were taken for the determination of nitrogen (Kjeldahl method), dry weight and total lipid (ether soluble material) (Sarett and Jandorf, '47) and ash.

In a third experiment, animals were maintained on either the fructose, glucose or dextrin diets for 7 months after injection with alloxan and sacrificed for analysis. The carcasses were analyzed as above, and the livers were minced and analyzed for solids and lipids (Sarett and Jandorf, '47) and

samples of the dried fat-free liver taken for nitrogen analysis by the Kjeldahl method.

For histologic study, blocks of liver were fixed in Carnoy's fixative, stained with periodic acid Schiff reagent for glycogen and with hematoxylin and eosin for tissue detail. Other blocks of liver were fixed in neutralized 10% formalin and frozen sections were stained with Sudan III for lipid determination. Liver, pancreas, kidney and adrenal tissues were fixed in Zenker's formol fixative and stained with hematoxylin and eosin. Kidney and pancreas were also fixed in 80% alcohol and stained with Best's carmine stain for glycogen. Part of the pancreas was fixed in Gomori's modification of Bouin's solution and stained with Gomori's chrome alum and hematoxylin to differentiate the beta cells of the islets of Langerhans.

RESULTS

The weight gain, blood sugar, food and water intake, urine volume and sugar excretion of the rats on the fructose and glucose diets in the first experiment are summarized in the first part of table 2. The differences between findings on the fructose and glucose diets were approximately the same in the two groups of rats. The change of carbohydrate had little or no effect on weight gain or on the level of blood sugar. The food intake was only slightly less on the fructose diet than on the glucose diet but there was a markedly lower water intake on the fructose diet, resulting in a decreased ratio of water to food intake. The urine volume was much smaller on the fructose diet than on the glucose diet and followed the water intake closely. The changes in urinary carbohydrate paralleled those in urine volume. On both glucose and fructose diets it was found that about 10 to 14 ml of urine was excreted per gram of carbohydrate in each animal, regardless of the degree of glycosuria.

The changes in the average volume of urine and amount of urinary carbohydrate excreted by these animals during the experiment are shown graphically in figure 1. In both groups the change from the glucose to the fructose diet resulted in

TABLE 2

Average daily intake and excretion in alloxan-diabetic rats maintained for successive periods on glucose and fructose diets (experiment 1) or on dextrin diet followed by glucose or fructose diets (experiment 2)

DIET	DAYS	AVERAGE BLOOD SUGAR	GAIN IN WEIGHT	FOOD INTAKE	WATER INTAKE	WATER INTAKE / FOOD INTAKE	URINE VOLUME	URINE CARBOHYDRATE
		mg/100 ml	*gm/day*	*gm/day*	*ml/day*	*ml/gm*	*ml/day*	*gm/day*
Experiment 1								
Group I (6 rats)								
Glucose	1–28	450	1.5	27	169	6.2	161	13.9
Fructose	29–49	419	1.9	26	134	5.1	125	10.9
Glucose	50–70	440	0.7	31	202	6.5	193	14.8
Group II (4 rats)								
Fructose	1–28	465	1.2	25	135	5.3	122	12.0
Glucose	29–49	456	1.1	30	196	6.5	184	15.0
Fructose	50–70	460	0.9	29	169	5.8	156	12.2
Experiment 2 (10 rats per group)								
Dextrin	1–12	441	0.4	28	186	6.7	174	15.0
Glucose	13–45	460	0.6	31	205	6.6	195	16.6
Dextrin	1–12	477	0.5	28	199	6.8	174	14.9
Fructose	13–45	450	1.0	27	151	5.5	136	12.2

a marked decrease in urine volume and urinary carbohydrate. (There was no decrease in food consumption or evidence of diarrhea on the fructose diet to account for these findings.) Following this a gradual increase took place until the excretion values found on the fructose diet reached a plateau at a level approximately two-thirds to three-fourths of those found on the glucose diet. The reverse change from

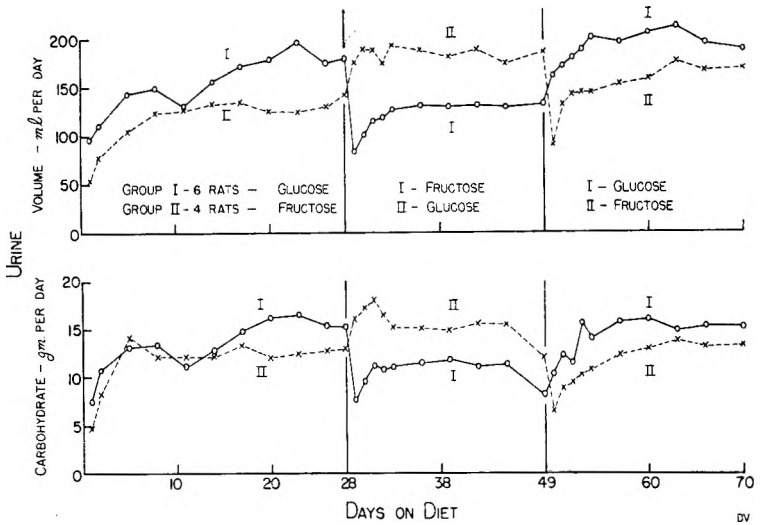

Fig. 1 Urine volume and carbohydrate excretion in alloxan-diabetic rats during successive periods on glucose and fructose diets (experiment 1).

the fructose to glucose diet was accompanied by an increased excretion of carbohydrate and of urine.

The results of the metabolic studies in the second experiment are shown in the lower half of table 2. On the dextrin diet the rats in each group showed essentially the same average water and food intake, water to food ratio, and urinary excretion findings. In the group changed to the glucose diet, all of these values either remained about the same as they were on the dextrin diet or increased slightly. In the group changed from the dextrin to the fructose diet, the food intake remained about the same but the water intake, the

ratio of water to food, the urine volume and urinary carbohydrate were markedly decreased. The average blood sugar of the animals on the fructose diet was a little lower than it was on the dextrin diet, while the blood sugar of the animals on the glucose diet was slightly higher than that found on the dextrin diet. These differences are not significant. There was also a small difference between the weight gains of the animals on the glucose and fructose diets.

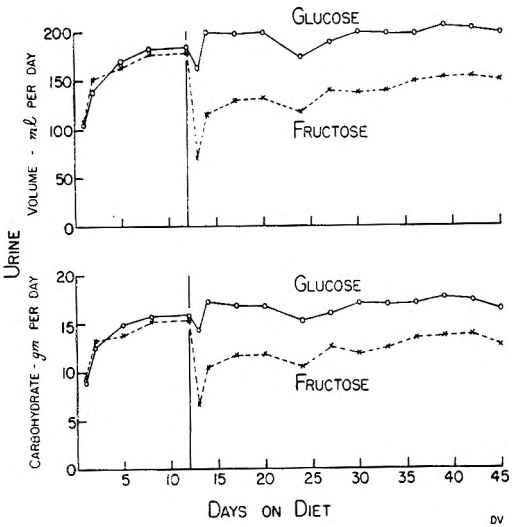

Fig. 2 Urine volume and carbohydrate excretion in alloxan-diabetic rats during successive periods on dextrin diet (12 days) and either glucose or fructose diet (experiment 2).

The average urine volume and urinary carbohydrate excreted throughout this experiment are plotted in figure 2. The transfer of the rats from their usual cages to the cooler environment of open-mesh metabolism cages may have been responsible, in part, for the low excretion values on the dextrin diet at the start of the metabolic studies. Ingle et al. ('53) have reported a decrease in glycosuria in diabetic rats exposed to cold. After stabilization on the dextrin diet, the change to the glucose diet resulted in a slight increase in urine volume and urinary carbohydrate, whereas the change

from dextrin to fructose resulted in a marked decrease in urine volume and urine carbohydrate. The urinary excretion gradually increased on the fructose diet and reached a plateau approximately 75% of that found in the glucose-fed animals. The lower urine volume and urine carbohydrate found on the fructose diet as compared with the dextrin diet was observed in every one of the 10 animals, and ranged from 17 to 67 ml

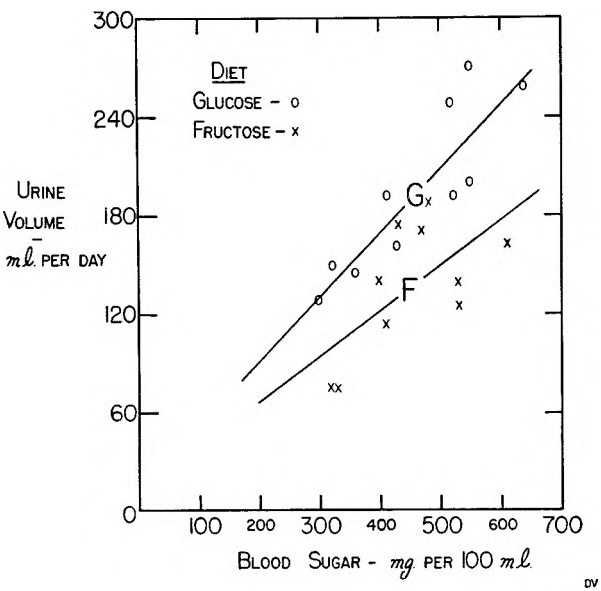

Fig. 3 Relation of urine volume to blood sugar level in alloxan-diabetic rats of experiment 2 maintained for 33 days on glucose and fructose diets. G and F represent the mean values for the rats on the glucose and fructose diets, respectively.

per day in volume and from 1 to 7 gm per day in reducing sugar. Only a small portion of the carbohydrate excreted by the animals receiving the fructose diet was fructose. This varied from 0.3 to 0.8 gm per day, averaging 0.6 gm per day, or from 2.7 to 6.8% of the total carbohydrate excreted, an average of 4.7%.

The blood sugar levels, which may be an index of the severity of the diabetes, can be correlated with the extent of

glycosuria and polyuria in these rats. (All blood samples were drawn in the morning.) In figure 3 the average daily volume of urine excreted by each rat is plotted against the average blood sugar level for that animal during the experiment. The average blood sugar level for the 10 rats receiving glucose was 460 mg per 100 ml and for those receiving fructose, 450 mg per 100 ml. Calculation of regression lines shows that the data for the animals receiving the glucose and

TABLE 3

Body composition of control and alloxan-diabetic rats of experiment 2

	CONTROL (4 RATS)	ALLOXAN DIABETIC [1] (10 RATS)	ALLOXAN DIABETIC [1] (10 RATS)
	Dextrin diet	Glucose diet 33 days	Fructose diet 33 days
Weight — gm	409	280	278
Fasted weight — gm	390	263	260
Carcass			
% Solids	39.3	33.2	33.7
% Lipid	14.7	7.4	8.4
% Protein	21.6	22.4	22.0
% Ash	3.9	3.9	4.0
Liver			
Weight — gm	10.5	10.4	12.5
% Body weight	2.7	4.0	4.8
Liver glycogen			
% Liver	0.6	2.9	3.1
Mg/100 gm Body weight	16.4	118	154
Kidneys			
Weight — gm	2.6	3.2	3.5
% Body weight	0.7	1.2	1.3
Adrenal			
Weight — mg	59.7	50.3	59.5
Mg/100 gm Body weight	15.5	19.4	23.1

[1] The diabetic animals received the dextrin diet for 105 days following alloxan injection, and either the glucose or fructose diet for the subsequent 33 days. The control rats (no alloxan) received the dextrin diet throughout the entire study.

fructose diets fall along two separate lines whose regression coefficients are significant (glucose diet, $P < 0.001$; fructose diet, $P < 0.05$). There is no significant difference between the slope of the regression lines calculated for the group on each diet but there is a highly significant difference between the adjusted means as calculated by the analysis of covariance ($F = 18.8$, $P < 0.001$) (Snedecor, '46). When diabetic animals with the same levels of blood sugar are compared, those receiving the fructose diet are found to excrete less urine and less sugar than those receiving the glucose diet. The change to the fructose diet did not markedly influence the level of blood sugar in the rat but resulted in a decreased loss of urinary carbohydrate by the animal. This was probably due to the difference in pathways of fructose and glucose metabolism.

Data on organ weights, liver glycogen and carcass composition in these animals are summarized in table 3. Control rats of the same age (no alloxan) which had been maintained on the dextrin diet throughout the experiment weighed more and contained a higher per cent of body lipid than did the diabetic animals. This difference in lipid accounts for the difference in per cent of solids found between the control and alloxan diabetic rats. There were no differences in levels of protein or ash. The kidneys of the diabetic animals on both diets were much larger both in terms of absolute weight and as related to body size than were those of the control animals. The adrenal glands were approximately the same weight in control and diabetic animals. The livers of the diabetic animals on the glucose diet weighed approximately the same as did those of the control animals but comprised a larger per cent of the body weight. The livers of the animals on the fructose diet were larger than those in both of the other groups and comprised 4.8% of the body weight which was significantly higher than the 4.0% found in the glucose group. The livers of normal rats kept on a diet containing fructose have also been found to be larger than those of rats on a diet containing glucose (Bachmann et al.,

'38). After the 24-hour fast the level of glycogen in the livers of the diabetic rats was much higher than it was in the normal animals. This elevated level of liver glycogen can be correlated with the high level of blood sugar and was only slightly higher in the animals receiving the fructose diet than in those receiving the glucose diet. When calculated in terms of milligrams per 100 gm of body weight, the liver glycogen of the animals receiving fructose was appreciably higher than that of the rats receiving glucose, because of the difference in liver weight.

Histologic study of the livers [2] of the diabetic rats showed normal tissue detail. Moderate amounts of lipid were observed in the control livers but not in those of the diabetic animals. No differences were observed between the livers of the glucose- and fructose-fed animals which might account for the larger size of the livers of the fructose-fed rats.

In the pancreas of the diabetic rats, the number and size of the islets of Langerhans and the number of beta cell granules were decreased as compared with normal. In the diabetic animals receiving glucose there were vacuolated areas in the cytoplasm of the acinar cells which were not found in the fructose-fed diabetic animals or in the control animals. No glycogen deposits were found in the islets or in the acinar cells of either the diabetic or the control rats. In the adrenal glands, vacuoles in the zona fasciculata indicative of lipids were more predominant in the control animals than in the diabetic animals. Kidney sections of the diabetic animals contained glycogen deposits in the proximal convolutions of the tubules and in the loops of Henle. During the course of the experiment cataract developed in one or both eyes of most of the diabetic rats.

In the third experiment, 11 rats which had been diabetic for 203 days and 4 control rats were sacrificed for analysis. The three diabetic rats which received the dextrin diet

[2] Histologic studies of the livers were kindly carried out by Dr. Sarah Luse, Department of Pathology, Western Reserve University School of Medicine. The other tissues were prepared and examined by D. L. Schneider in our laboratory.

throughout the experiment had much lower blood sugars throughout the experiment than the 8 which received the glucose or fructose diets for the last 98 days (table 4). The differences in per cent of carcass solids among the groups were found to be related to differences in lipid content. The control animals contained 15.9% body lipid, the mildly diabetic animals on the dextrin diet, 9.7%, and the severely diabetic animals on the glucose diet only 4.9%. Animals with comparable high levels of blood sugar which were receiving the fructose diet contained 8.0% lipid. In the previous ex-

TABLE 4

Body composition of control and alloxan diabetic rats of experiment 3

	CONTROL (4 RATS)	ALLOXAN DIABETIC (3 RATS)	ALLOXAN DIABETIC [1] (4 RATS)	ALLOXAN DIABETIC [1] (4 RATS)
	Dextrin diet	Dextrin diet	Glucose diet 98 days	Fructose diet 98 days
Weight — gm	416	306	273	275
Fasted weight — gm	402	293	251	255
Blood sugar —				
mg/100 ml	135	277	502	456
Carcass —				
% Solids	40.2	36.9	31.8	35.0
% Lipids	15.9	9.7	4.9	8.0
% Protein	20.8	22.3	22.4	22.4
Liver —				
% Body weight	3.0	4.3	3.9	4.6
% Solids	29.6	29.5	29.5	30.0
% Lipid	6.6	4.5	3.2	2.3
% Protein	21.9	22.1	21.6	22.9
Kidneys —				
% Body weight	0.6	1.0	1.3	1.5
Adrenal glands —				
mg/100 gm	15.1	19.1	20.3	23.4

[1] These animals received the dextrin diet for 105 days following alloxan injection, and either the glucose or fructose diet for the subsequent 98 days. The other rats, normal controls and alloxan diabetic rats, received the dextrin diet throughout the entire study.

periment (table 3), the level of lipid in the carcasses of the fructose-fed diabetic animals was also slightly higher than that found in the glucose-fed rats.

The livers of the animals in these groups contained similar levels of protein and of total solids (table 4). The highest level of liver lipids was found in the control rats and the lowest in the fructose-fed diabetic rats. The level of liver glycogen probably differed in the 4 groups and may account for the fact that the difference between total solids and the sum of protein and lipids varied among the groups. The data on kidney and adrenal weights agree with the findings in table 3.

DISCUSSION

The decrease in glycosuria and polyuria which is found when fructose replaces glucose in the diet of alloxan-diabetic rats is not fully understood but may be due to the more rapid uptake of fructose than of glucose by the body tissues. This interpretation is supported by the fact that fructose comprices only 3 to 7% of the carbohydrate excreted by the rats on the fructose diet. Differences between the initial pathways of glucose and fructose metabolism are known which account for the more rapid utilization of fructose than of glucose, especially in the diabetic animal. Glucose is converted to glucose-6-phosphate by the action of hexokinase, while fructose may be converted to fructose-6-phosphate by hexokinase or to fructose-1-phosphate by fructokinase (Vestling et al., '50; Cori et al., '51; Hers, '52; Mackler and Guest, '53). Insulin affects the action of hexokinase but has no effect on fructokinase (Mackler and Guest, '53). Liver slices of alloxan-diabetic rats oxidize fructose at a normal rate, and utilize glucose very poorly (Chernick and Chaikoff, '51). In diabetic subjects, utilization of intravenously administered fructose is normal, whereas use of glucose is impaired (Craig et al., '51; Miller et al., '52b).

The advantages of intravenous fructose over glucose in diabetic subjects have been shown to persist throughout a 9-day study (Miller et al., '52a). However, when oral fruc-

tose was administered to diabetic subjects for prolonged periods, it appeared to be well utilized at first, but after some time hyperglycemia and glycosuria usually followed its use (Joslin, '46). This observation seems to be similar to that seen in the present experiments on alloxan-diabetic rats in which the use of fructose in the diet resulted in an initial marked decrease in polyuria and glycosuria, which slowly returned to higher levels. However, the levels of polyuria and glycosuria reached and maintained with fructose in the diet were significantly lower than those found with glucose (figs. 1 and 2).

Mayer et al. ('53) have reported that in mice with the hereditary obesity-diabetes syndrome, a diet containing fructose suppresses glucosuria but does not prevent the weight increase. In the present study the fructose diet suppressed the glycosuria and polyuria and had little or no effect on the level of blood sugar.

The high level of liver glycogen found in alloxan-diabetic rats following a 24-hour fast has been reported previously (Morita and Orten, '49; Teng et al., '52). Although this increased level of glycogen may result from the stress imposed by the alloxan diabetes, it is more likely the direct result of the high blood sugar level. In the livers of diabetic patients dying in diabetic coma, an elevated level of liver glycogen has also been found (Vallance-Owen, '52).

SUMMARY

In alloxan-diabetic rats maintained on a diet containing either fructose or glucose, the following observations have been made:

1. Diabetic animals kept on a fructose-containing diet excrete less urine and less urinary carbohydrate than do the same animals on a glucose-containing diet or other animals with comparable levels of blood sugar on the glucose diet. Approximately 5% of the sugar excreted by the diabetic animals receiving the fructose diet is fructose.

2. Diabetic animals on the diet containing fructose consume slightly less food and markedly less water than do comparable animals on the glucose-containing diet.

3. The livers of the diabetic animals receiving fructose are significantly larger than those of the diabetic animals receiving the glucose diet. The kidneys of the diabetic rats on both diets are larger than those in control animals.

4. Histologic study shows normal tissue detail in the livers of the diabetic animals. In the pancreas the number and size of the islets and the number of beta cell granules are markedly decreased in the diabetic animals. In the kidneys of the diabetic rats glycogen deposits are found in the proximal convolutions of the tubules and the loops of Henle.

5. Alloxan-diabetic rats are smaller and contain a lower per cent of lipids in the liver and carcass than do control animals.

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SEQUENCE OF HISTOLOGIC CHANGES IN SKIN OF DOGS IN RELATION TO DIETARY FAT[1]

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THREE FIGURES

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Previous studies on the histologic structure of the skin of dogs disclosed that absence of fat in the diet effects definite alterations in the epidermis and dermis, including the hair follicles, sebaceous glands and capillaries (Hansen, Holmes and Wiese, '51). In order to understand better the nutritional significance of dietary fat in relation to the condition of the skin, a more detailed study of the sequence of cellular changes seemed warranted. The present report covers the findings of further work which has been carried out on a group of 8 litter-mate puppies and on another group of 4 litter-mate dogs, 10 months old.

MATERIAL AND METHODS

Experimental conditions for maintaining the animals were the same as those previously described (Hansen and Wiese, '51). Of the group comprising the 8 litter-mate puppies, at 6 weeks of age 4 were given the control diet containing 29% of the calories as fresh lard and 4 were placed on the low fat diet having 1% of the calories as fat. In the second group of 4 litter-mate dogs which had been maintained on the

[1] This work was supported in part by the U. S. Department of Agriculture through a contract sponsored by the Bureau of Human Nutrition and Home Economics.

low fat diet for 9 months and showed typical signs of fat deficiency, three were given diets in which fat calories were substituted for 29% of those from sucrose. The fats which were used were fresh lard, cocoa butter and an oil emulsion. These contained varying amounts of unsaturated fatty acids. The 4th dog remained on the low fat diet.

At 4-week intervals, skin biopsies of each animal were taken from the dorsal aspect of the thigh and from the ventral surface of the chest. Microscopic sections were prepared from blocks of skin fixed in Bouin's solution, the skin being oriented alike each time. Uniformity of all stages of preparation was maintained and the 6-μ sections were stained with hematoxylin and eosin. Unless frozen sections were to be studied, we saw no advantage in other staining techniques.

RESULTS

Control puppies

In the skin of healthy control puppies receiving 29% of their calories as fresh lard, the epidermis is two to three cells in thickness, with the outer layer flattened to a granulosum type. A few basophil granules are present. Above this layer is a thin lace-like stratum disjunctum of keratinized material with hardly a trace of stratum lucidum. The basal layer of the stratum germinativum is low columnar to cuboid with little mitosis. The cells are not swollen and hence the spinous processes are not evident. The lower surface of the epidermis is quite smooth and rests on a rather homogeneous collagenic layer of dermis containing a few fibroblasts, capillaries and nerve fibers. The hairs are firm picric-stained rods which gradually attain an eosinophil casing as they pass into the follicle. There is no loose keratin in the shaft. Around the large hairs there is a group of smaller hairs; all are connected with a single outlet. At the level of the stratum reticularis, sebaceous glands are bunched on the sides and lower slope of the follicle. They are not large but show a complete gradation from germinative to degenerative cells

with little sign of a lumen in any gland. Just below this level may be found a solid cord of chromatic cells which connect with the side of the follicle and course downward in an undulating fashion. They end in a rather small alveolar type of gland. This sudoriparous gland is backed completely by epitheliomuscular cells. Normally the lumen is half as wide as the diameter of the gland.

Puppies on low fat diet

When young puppies are placed on a diet having 1% of the calories as fat, the first histologic change in the skin seems to consist of an increased water content of the cells and matrix so that the cells swell. For a while there is evidence of stimulation as indicated by an increase in the number of cells in the epidermis and hair follicles and by increased activity of the sebaceous and sudoriparous glands.

The epidermis is thickened and is similar in appearance to that of the palms and soles of human subjects. The intercellular spaces widen so that intercellular bridges are prominent. The basal layer of cells becomes cylindrical and the base line is very uneven. This general effect is known as "pegging." The nuclei in this lamina become chromatic and often pyknotic. Mitosis is shifted to the layers immediately above. The stratum granulosum includes two layers or more. The keratinization process is slowed down so fragments of nuclei may be seen in the desquamatum. The fluid oozing from the epidermis holds the keratinized material together, forming a basal plaque or stratum lucidum.

The entire dermis becomes thickened due to the accumulation of fluid. The stratum reticularis appears somewhat less fibrous than in the control animals and there is infiltration of cells of monocytic type from the capillaries. The invading cells have abundant cytoplasm and nuclei which are very much smaller than those of fibroblasts. The cells are not phagocytic. As the number increases, there is a definite breakdown and invasion of the adjacent epidermis at separated

points. When the infiltrated cells reach the exterior surface of the skin, there is evidence of inflammatory changes and the presence of neutrophil polymorphs. The basophil cells of the hair follicles become larger than those found in control animals and lose their basophil stain. The hair shafts become coated with keratinized sheets of loose material and the shaft shrinks in size. Later it may show fragmentation. On the low fat diet the individual cells of the sebaceous glands swell but do not increase in number. The secretory cycle continues by cell degeneration (holocrine secretion).

A very marked change in the appearance of the skin of puppies on the low fat diet concerns the deeper glands which are modified sweat glands. They extend down along the hair follicles and may show 5 or 6 coils. The ducts open up and the glands become active. The lumen increases and the epitheliomuscular cells become very numerous and prominent. The individual cells become low cylindrical instead of cuboid. The inner ends are bulbous and seem to contribute blebs of material by an apocrine mode of secretion into the hair follicles. They do not reach the surface of the skin. The sweat glands are usually rudimentary in the control animals but show some variation with age. In young puppies there are solid cords of chromatic material which later develop into long ducts without secretory tubules.

It is with a remarkable degree of uniformity that the sequence of these changes occurs in the epidermis and dermis. The most apparent change is in reference to the increasing thickness of the epidermis as the animal shows increasing signs of fat deficiency. However, other alterations occur simultaneously, so that if one observes an increase in the number of cell layers in the epidermis, one also finds variable degrees of change in the other structures. To assist in following the sequence of these changes, the designations + to ++++ are used to indicate the degree of histologic change described in table 1. Photomicrographs of the skin of a control dog and of animals showing various degrees of change

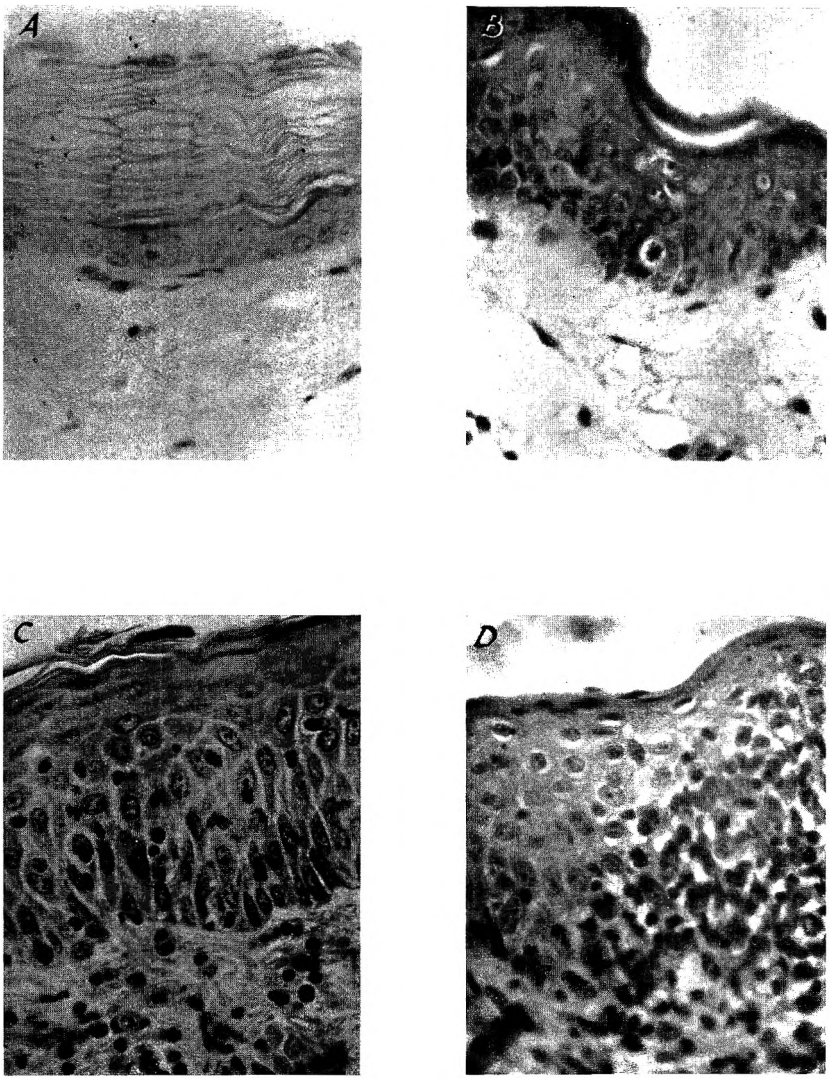

Fig. 1 Photomicrographs of epidermis of dogs, high power (8 × 43).

A. Control diet.

B. Low fat diet, stage ++, note mitosis.

C. Low fat diet, stage +++, note cell infiltration.

D. Low fat diet, stage ++++, note ulceration.

are presented herewith: epidermis in figure 1, hair follicles in figure 2, sudoriparous glands in figure 3.

Rate of development of histologic changes

The maximum histologic changes were demonstrable within about 6 months in the young puppies receiving the low fat diet. Biopsies taken from the region of the chest and thigh showed parallel changes in all preparations. Using the designations C and + to ++++, as described in table 1, the rate of development of the skin changes in the 4 litter-mates on the low fat diet is indicated in table 2.

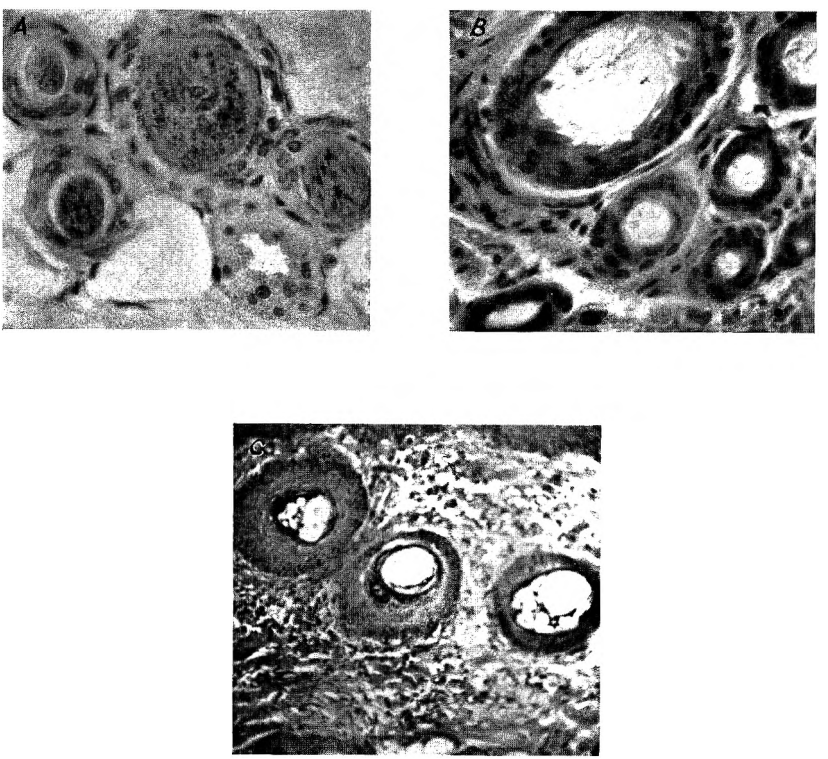

Fig. 2 Photomicrographs of hair follicles.
A. Control diet, with normal hair shafts, high power (8 × 43).
B. Low fat diet, stage ++, high power (8 × 43).
C. Low fat diet, stage ++++, low power (8 × 10).

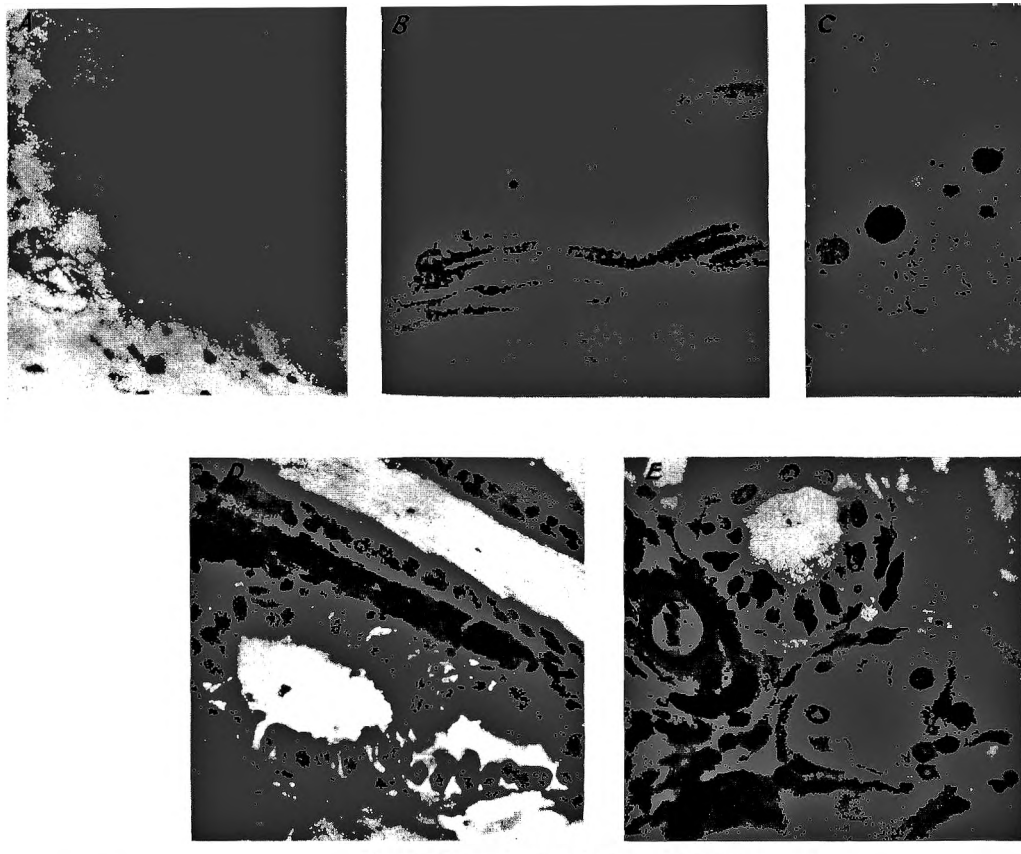

Fig. 3 Photomicrographs of sudoriparous glands.

A. Control diet, young animal, note solid cords of chromatin material which precedes formation of ducts, high power (8 × 43).
B. Control diet, mature animal, note long ducts without secretory tubules, low power (8 × 10).
C. Low fat diet, stage ++, early secretory stage, note coils at base of hair follicles, low power (8 × 10).
D. Low fat diet, stage +++, note apocrine secretion, high power (8 × 43).
E. Low fat diet, stage ++++, details of sebaceous and sudoriparous gland structures, note complete investment of sudoriparous gland by muscular cells.

TABLE 1

Sequence of changes in histological structure of skin of dogs on low fat diet compared with control animals

	C (control)	+	++	+++	++++
Epidermis					
Cell layers	2–3	2–4	4–5	6–7	8–10
S. disjunctum	lace-like	lace-like	lucidum	sticky	thick, sticky
S. granulosum	few granules	granules	marked granules	2 cell layers	3 cell layers
S. spinosum	no spaces	cells swollen	cells swollen	spaces & processes	same
S. basalis	uniform cuboid	mitosis	more mitosis	pegged, irregular	pegged, undulating
Infiltration	none	none	none	some round cells	heavy in spots, ulceration
Uniformity	uniform	uniform	uniform	irregular base	irregular both surfaces
Swelling	none	slight	some	considerable	extreme
Dermis					
Cell types					
Fibroblasts	few	present	more	same	same
Monocytes	none	occasional	some	more plus small round cells	same
Polymorphs	none	none	none	in veins	some in tissues
Matrix					
Tissue spaces	none	none	none	small in papillary layer	many in papillary layer
Hair follicles					
Hair shaft	solid	solid	solid	shrunken	fragmented
Loose keratin	none	some	sheath surface	sheath and hair	fills follicles
Sebaceous glands					
Size	small	swollen	large	large	variable
Activity	no lumen	no lumen	no lumen	lumen	atrophic cells
Sudoriparous glands					
Ducts	long solid	longer	long as gland	shorter than gland	very short, some atrophy
Gland length	short, deep	longer	doubles	undulating	full length
Activity	low cells	cuboidal	high cells	high cells	variable
Lumen	small	increased	large	large	variable

TABLE 2

Rate of development of histologic changes of skin of puppies receiving a low fat diet

DOG NO.	BIRTH	DIET		DEGREES OF CHANGE				
				Weeks on diet				
		Low fat	Fat	10	14	18	22	25
77	11/1/51	12/1/51	none	++	++	++	+++	+++
78	11/1/51	12/1/51	none	+++	+++	++	+++	+++
79	11/1/51	12/1/51	none	++	+++	++++	+++	++++
80	11/1/51	12/1/51	none	++	+++	+++	+++	+++
				8	12	16	20	23
81	11/1/51	12/1/51	29% cal. (lard)[1]	+[2]	C	C	C	C
82	11/1/51	12/1/51	29% cal. (lard)[1]	+[2]	C	C	C	C
83	11/1/51	12/1/51	29% cal. (lard)[1]	+[2]	C	C	C	C
84	11/1/51	12/1/51	29% cal. (lard)[1]	+[2]	C	C	C	C

[1] Started 12/14/51.
[2] Some residual effects of previous low fat diet evident, particularly hydration of skin and elongated sudoriparous glands.

TABLE 3

Rate of reveral of histologic changes in skin of fat-deficient dogs after receiving dietary fat

DOG NO.	BIRTH	DIET		HISTOLOGIC APPEARANCE				
					Weeks after starting fat			
		Low fat	Fat 29% of cal. 1/31/52	Initially	6	10	14	18
71	4/1/51	5/12/51 to 1/30/52	Lard	++++	++	++	C	C
72	4/1/51	5/12/51 to 1/30/52	Lipomul	++++	+++	++	++	+[1]
73	4/1/51	5/12/51 to 1/30/52	Cocoa butter	+++	++	+++	++	+[2]
74	4/1/51	5/12/51 to 6/6/52		++++	++++			

[1] Epidermis and dermis back to "C" but sebaceous and sudoriparous glands are not.
[2] Skin same as no. 72 but ears still running.

Sequence of recovery from fat-deficient state

The 4 litter-mate dogs which had been maintained on the low fat diet for 9 months showed the typical signs of fat deficiency. Six weeks after isocaloric substitution of 29% of the sucrose calories by fat (lard, an oil emulsion [2] or cocoa butter), the skin of the three animals receiving fat showed definite improvement but all histologic structures did not return to the condition of those of the control puppies at a uniform rate.

The water content of the skin was reduced very soon and the epidermis with its disjunctum changes recovered first. Changes in the dermis took place much more slowly. Monocytes persisted for some time and although the keratin-filled hair follicles cleared rapidly, the hair structure itself was delayed in recovery. The cells of the sebaceous glands shrank gradually. During the observation period of 18 weeks, the sudoriparous glands slowly became quiescent but the glands did not return to the normal length nor did the duct system lengthen to its previous normal extent.

There was a definite variation in the rate of recovery of the histologic structures of the skin with the dietary fat given. Histologic restoration was most rapid in the animal which received 29% of its calories from fresh lard. This is in keeping with previous observations on the gross appearance of the skin of dogs fed lard or baconfat (Hansen and Wiese, '51). The poorest response was with cocoa butter.

Using the same desigations of C and + to ++++ as a measure of the histologic structure of the skin of the 4 litter-mate dogs, the rate of recovery from the fat deficient state is shown in table 3.

DISCUSSION

It will be noted in table 1 that only at an early stage in the sequence of changes was there evidence of increased mitotic activity; e.g., when the epidermis was beginning to increase in thickness (stage ++). This is an especially significant

[2] Lipomul.

observation, since thereafter the cell layers in the epidermis might double in number from 4–5 to 9–10 without any evidence of an increase in mitosis. The logical explanation is the accumulation of epidermal cells due to failure of normal maturation. This is borne out further by the pyknotic appearance of the nuclei and evident alteration in the keratinization process.

A second pertinent observation is with respect to the invading cells in the dermis. At first these are principally mononuclear in type, stainable with both eosin and basic stains, but as the fat deficiency signs progress, small round cells may be found perforating the epidermis to such an extent as to appear ulcerated. Under such spots the dermis has numerous infiltrated cells among which neutrophil polymorphs may be identified. There is a possibility that these cell changes may be related to the observed increased susceptibility to infection of animals which lack fat in the diet.

A third marked finding is the increased activity of the sebaceous glands. The significance of this physiologic response is not clear, but it is reflected in the development of greasy skin and hair in fat-deficient dogs. It is probable that the chemical and physical nature of secreted sebum changes.

Another striking change relates to the sudoriparous glands. It is commonly stated that sweat glands in dogs are limited to toe pads and nose. However, the presence of sweat glands in dog skin has been observed since 1835 (Gurlt, 1835), and the most recent description of their activity as measured by the starch iodine method is described by Aoki and Wada ('51). Histologic evidence of increased activity of the sweat glands of rats fed low fat diets has not been noted (Williamson, '41; Panos and Finerty, '53; Ramalingaswami and Sinclair, '53). However, the latter authors point out that changes in the ducts of human sweat glands as well as follicular changes have been observed in phrynoderma. It was demonstrated early that rats on a low fat diet showed an increased metabolic rate (Wesson and Burr, '31). Kunkel and

Williams ('51) found that in the fat-deficient rat the hepatic cytochrome oxidase activity is increased, suggesting that this enzyme is related to the metabolic rate. It may be conjectured that increased activity of the sudoriparous glands is an attempt to compensate physiologically for an increased heat production. A human subject on a low fat diet also showed an increased oxygen consumption (Brown, Hansen, Burr and McQuarrie, '38).

There are no features which are suggestive of hypersensitivity or of a deranged immunologic phenomenon's being responsible for the histologic changes. The matter of a possible association with the metabolism of vitamin A has arisen on a number of occasions. In our animals not only was there a high dietary intake of vitamin A, but the blood levels also were high and in the same range for both control and low fat animals (Hansen and Wiese, '51). Ramalingaswami and Sinclair ('53) have described the histologic features of the skin of rats in a state of both vitamin A and essential fatty acid deficiency and found the histologic pictures to be distinctly different. These workers were especially concerned with phrynoderma in man. They observed that the skin changes in essential fatty acid deficiency in rats were closely similar to those in phrynoderma, whereas the histologic changes in vitamin A deficiency bore only a slight resemblance to those in phrynoderma.

There seems to be little doubt that the chemical, physiologic and histologic changes which are observed following prolonged use of a low fat diet are due to the lack of unsaturated fatty acids, which are not synthesized by various animal species in sufficient amounts for storage to meet their needs for these fatty acids.

The question arises as to whether it is possible to gain insight into the fundamental mechanisms involved by a consideration of the sequence of histologic changes which occur in the skin structures. It is conceivable that specific unsaturated fatty acids serve as an integral part of protoplasmic structure; e.g., are linked with protein as a lipoprotein or

combined with prosthetic groups to function in enzyme systems. Since the histologic changes are reversible, one is led to believe from the sequence of restoration that linoleic acid supplies a necessary component for the structure of certain cells which permits them to complete their life cycle.

SUMMARY

Periodic histologic examinations of the skin were made on 8 young puppies, 4 of which were developing deficiency symptoms due to lack of dietary fat, and on 4 adult dogs, three of which were recovering from the fat-deficient state after the addition of fat to the diet.

Rather remarkable was the uniformity of the changing microscopic appearance of the skin both during increasing deficiency states and during recovery. Alterations in the stratum corneum, collagen structure, cellular infiltrate of the dermis, hair follicles, sebaceous glands and sudoriparous glands, all were related to changes in the epidermis, particularly with regard to the number of cell layers. More rapid restoration of the microscopic structure occurred when the dietary fat was high in unsaturated fatty acid content.

The findings suggest that dietary fat supplies a factor necessary for the maturation of epithelial, sebaceous and sudoriparous cells which, when absent from the diet, results in distinct abnormality in the skin demonstrable both microscopically and grossly. The process is readily reversible and changes in an orderly fashion when fat is added to the diet.

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SOME PROPERTIES OF THE CHICK GROWTH INHIBITOR IN LINSEED OIL MEAL

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ONE FIGURE

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Linseed meal has been shown to depress the growth of chicks unless it has been treated with water, allowed to stand a few hours and then dried before mixing in the ration (Kratzer, '46, '47; McGinnis and Polis, '46; MacGregor and McGinnis, '48). It was shown subsequently that the addition of pyridoxine to the ration containing linseed oil meal would improve the growth of chicks although the ration originally contained more pyridoxine than is usually required for optimum growth (Kratzer and Williams, '48).

Further experiments have been conducted to obtain more information on the nature of the growth inhibitor in linseed oil meal and factors which influence its destruction or counteraction.

EXPERIMENTAL

The various linseed oil meal samples and preparations from linseed oil meal were mixed in rations for chicks at a 30% level. "Old process" (produced by hydraulic or expeller methods) linseed oil meal was used except in experiments testing solvent process linseed oil meal and linseed grits. The basal ration was essentially that described previously (Kratzer and Williams, '48) except that the vitamin supplement contained thiamin hydrochloride, 1.0 mg; riboflavin, 2.0 mg; calcium pantothenate, 6.0 mg; niacin, 9.0 mg; inositol,

1.0 gm; and choline chloride, 1.0 gm per kilogram of ration. Pyridoxine · HCl was added to certain diets at the level of 35 mg per kilogram of ration.

S. C. White Leghorn cockerel chicks were kept on a practical ration for about a week before being segregated into groups to be fed the experimental rations. The chicks were kept in heated batteries with raised wire floors and were

TABLE 1

Effect of autoclaving, heating and water treating of "old process" linseed oil meal upon its chick growth promoting value in the absence and presence of added pyridoxine

SUPPLEMENT	PYRIDOXINE ADDED	PERCENTAGE DAILY GAIN				AVERAGE
		Exp. 1	Exp. 2	Exp. 3	Exp. 4	
Untreated linseed meal	—	2.03	2.89	0.87	1.04	1.7
Untreated linseed meal	+		5.00	3.84		4.4
Autoclaved linseed meal	—	5.15 [1]	4.47 [1]	3.23 [1]	4.97 [2]	4.5
Autoclaved linseed meal	+		5.40 [1]	3.70 [1]		4.6
Heated linseed meal	—			1.79	1.10	1.4
Heated linseed meal	+			3.64		3.6
Water treated linseed meal	—	5.37	5.12	3.46	4.92	4.7
Water treated linseed meal	+		5.83	4.12		5.0
Duration, days		7	21	26	14	
Number chicks/group		4	10	7	6	

[1] — 30 minutes.

[2] — One hour.

supplied feed and water ad libitum. The experiments were of from 7 to 26 days' duration.

Effect of heat and water treatment

Since previous work has shown that water treatment was effective in destroying the growth inhibitor, it seemed important to determine whether this was due to enzymatic, microbiological, or chemical action. By autoclaving linseed oil meal one could destroy enzyme activity and also prevent microbiological action. Therefore, if autoclaving were found effective in destroying the growth inhibitor, one could assume

that the mechanism was by some way other than these two processes.

"Old process" linseed oil meal was autoclaved in shallow pans for 30 minutes or one hour at 15 pounds' pressure per square inch. Another sample was heated in an oven at the

TABLE 2

Effect upon the growth of chicks of pyridoxine supplementation or water treatment of linseed oil meal and grits, or both

LINSEED SUPPLEMENT	SAMPLE	PYRIDOXINE ADDED	WATER TREATED	PERCENTAGE DAILY GAIN			AVERAGE
				Exp. 5	Exp. 6	Exp. 7	
Solvent processed linseed	a	—	—	2.20	2.93		
Oil meal or flakes	b	—	—	2.21	2.87		2.6
	c	—	—			3.01	
	a	+	—	5.85	4.71		
	b	+	—	6.06	4.86		5.3
	c	+	—			5.05	
	a	—	+	5.52	5.13		
	b	—	+	4.33	4.89		4.9
	c	—	+			4.80	
	a	+	+	6.62	5.73		
	b	+	+	6.55	5.77		6.1
	c	+	+			5.72	
Linseed grits		—	—	3.68	4.55		4.1
		+	—	5.59	5.17		5.4
		—	+	4.72	5.24		5.0
		+	+	6.08	5.60		5.8
Duration, days				18	23	21	
Number of chicks/group				6	9	6	

same temperature for the same period of time. A third sample of the same meal was water treated as has been described previously (Kratzer, '47).

The results (table 1) indicate that the growth inhibitor may be destroyed by autoclaving but not by dry heat. Autoclaving seemed as effective as the water treatment in improving the nutritional value of the meal for chicks. These results are interpreted as strong evidence that the growth improve-

ment results from chemical destruction of the growth inhibiting factor rather than from heat, microbiological or enzymatic action.

Since a steam treatment is used in the manufacture of linseed grits, it was of interest to determine whether this product might be superior to other linseed products for chick rations. Solvent extracted linseed flakes and two samples of

TABLE 3

Effect of pyridoxine and aureomycin upon growth of chicks fed "old process" linseed oil meal or water treated linseed oil meal

LINSEED SUPPLEMENT	PYRIDOXINE	AUREOMYCIN	EXP. 8	EXP. 9	EXP. 10	EXP. 11	AVERAGE
Linseed oil meal	—	—	1.76	1.57	3.72	1.70	2.2
Linseed oil meal	+	—	4.10	4.99	5.11	4.33	4.6
Linseed oil meal	—	+	2.35	1.49	3.07	2.24	2.3
Linseed oil meal	+	+	5.85	4.88	5.05	5.13	5.2
Water treated linseed oil meal	—	—		5.20	5.14		5.2
Water treated linseed oil meal	+	—		5.66	5.30		5.5
Water treated linseed oil meal	—	+		5.17	5.19		5.2
Water treated linseed oil meal	+	+		5.75	5.20		5.5
Duration, days			13	16	21	19	
Number of chicks/group			8	10	7	11	

commercially produced solvent process linseed oil meal (samples a, b, and c respectively, table 2) were also tested by feeding (1) alone, (2) with added pyridoxine, (3) after water treatment and (4) after water treatment with added pyridoxine.

The solvent processed linseed oil meal and flakes gave growth approximately equal to that observed with old process linseed oil meal (table 2). Water treatment caused improvement nearly equal to that produced by pyridoxine supplementation. In every instance the combination of water treatment and pyridoxine gave growth slightly better than

each variable alone. The linseed grits without treatment were somewhat better than the solvent processed meals, but still responded to water treatment and pyridoxine supplementation.

Effect of aureomycin

There is evidence that antibiotics in the feed of chicks alter the microflora of the intestinal tract and in some cases have been shown to spare the need for vitamins in the diet (Biely and March, '51). A series of trials was conducted to determine whether aureomycin in the feed could partially

TABLE 4

Effect of injecting pyridoxine into chicks fed 30% untreated "old process" linseed oil meal

PYRIDOXINE ADMINISTRATION	AVERAGE GAIN		RELATIVE EFFECTIVENESS OF ADMINISTRATION TO FEEDING
	Exp. 12	Exp. 13	
	gm	*gm*	
None	24.0	16.7	
1 mg/day in leg	76.2		52%
5 mg/day in leg		40.8	53%
5 mg/day intraperitoneally		37.6	46%
5 mg/day orally		36.4	43%
37.5 mg/kg in feed	123.6	62.3	
Duration, days	21	15	
Number of chicks/group	5	8	

overcome the toxicity of linseed meal, by promoting the synthesis of pyridoxine in the intestinal tract. Crystalline aureomycin was added to various diets at a level of 50 mg per kg of ration.

Aureomycin was ineffective in improving growth of chicks fed untreated linseed oil meal although in two of the 4 trials it gave responses in the presence of pyridoxine (table 3). There was no response to aureomycin with water treated linseed oil meal, either in the presence or absence of pyridoxine. It thus seems evident that aureomycin does not stimulate synthesis of pyridoxine in the intestinal tract to an appreciable degree.

Injections of pyridoxine

A comparison was made of the effect of injected and dietary pyridoxine in counteracting the growth inhibitor. Assays by *Neurospora sitophila* (Stokes, '47) of the basal rations containing old process linseed oil meal and the same sample after water treatment gave approximately 15 μg of pyridoxine per gm in each ration. The control ration was supplemented

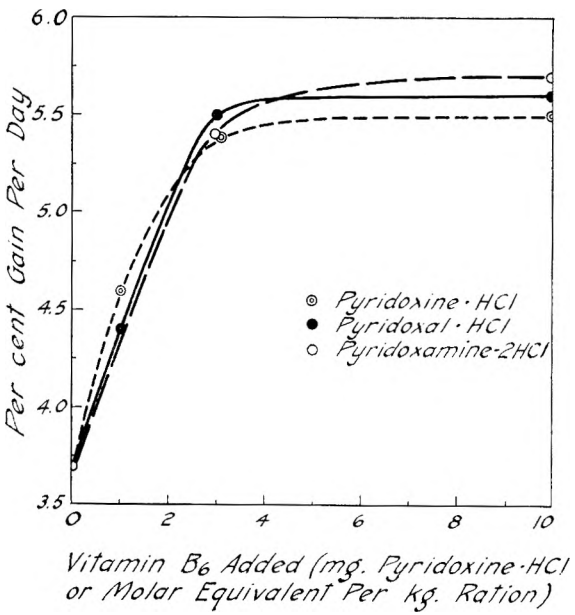

Fig. 1 Effect of various forms of vitamin B_6 upon the growth of chicks fed ration containing 30% linseed oil meal.

with 37.5 mg of pyridoxine per kg. If one assumed that a chick would eat 20 gm of feed per day, the daily pyridoxine intake of a chick in the basal group would be approximately 1 mg per day.

In experiment 12 chicks were given injections of 1 mg of pyridoxine daily in the leg muscle (table 4). In experiment 13 chicks received 5 mg per day injected either in the leg or intraperitoneally. Growth of the chicks in the injected groups

was approximately 50% of that in the control group in which pyridoxine was mixed in the feed. Oral administration of a pyridoxine solution equivalent to the injected dose gave roughly the same response as injection.

Forms of vitamin B_6

Sarma et al. ('46) have shown that when mixed in the ration, pyridoxal and pyridoxamine were less active than pyridoxine for the growth of chicks. When given orally the three compounds were equally effective on a molar basis. Waibel et al. ('52) showed that pyridoxine, pyridoxal and pyridoxamine were equally active in a chick ration containing autoclaved starch. These three vitamin B_6-active compounds were tested in a ration containing 30% linseed oil meal to determine their relative effectiveness in counteracting the growth inhibitor. Ten chicks per group were fed the experimental rations for a 21-day period.

The growth responses from pyridoxine · HCl, pyridoxal · HCl and pyridoxamine · 2HCl were very similar (fig. 1). This indicates that the vitamin B_6 compounds were being used directly by the chick rather than by microorganisms, since many of the latter show a differential response to the forms of vitamin B_6.

DISCUSSION

There is strong evidence for the existence of a specific growth inhibiting factor in linseed oil meal although isolation of such a factor has not been achieved. The factor is destroyed by water treatment and autoclaving but is not destroyed by dry heat. This tends to eliminate the possibility of the factor being destroyed by enzyme or microbiological action since these activities would be destroyed in the course of autoclaving. Pyridoxine has been found to counteract the factor consistently, although in many cases the combination of pyridoxine supplementation and water treatment was superior to either variable alone. This may possibly be explained by

a slight change in the physical texture of the meal during water treatment which makes it superior to the untreated meal.

Aureomycin was ineffective in improving the growth of chicks fed linseed oil meal. This shows that the destruction of the factor is not favored by the change in the microflora produced by this antibiotic. It also indicates that aureomycin does not stimulate synthesis of pyridoxine, at least in amounts to cause appreciable counteraction of the growth inhibitor. The fact that pyridoxine, pyridoxamine and pyridoxal are equally effective in alleviating the toxic factor further indicates that microorganisms in the intestinal tract are not involved in the mechanism by which the factor is destroyed.

Injection of pyridoxine into chicks fed linseed oil meal was only half as effective in counteracting the growth inhibitor as including the vitamin in the feed. Although the reason for this is not clear, it may be explained by the fact that with a single daily injection there is a large amount of pyridoxine available for counteracting the inhibitor for a short time only. Just previous to an injection the available pyridoxine might be reduced considerably and would not be sufficient to counteract the inhibitor ingested at that time. When mixed in the feed, the pyridoxine is available to the bird at the same time that the growth inhibitor is effective.

SUMMARY

Linseed oil meal depressed the growth of chicks when fed at a level of 30% of the diet. Water treatment and autoclaving were effective in destroying the growth depressing factor while dry heat was ineffective. Linseed grits, produced with some steam treatment, were less growth depressing than old process or solvent process meal, but still responded to water treatment or pyridoxine supplementation.

Aureomycin did not improve the growth of chicks fed linseed oil meal indicating that there was no stimulation of pyridoxine synthesis by the antibiotic.

Single daily doses of pyridoxine given orally or injected intraperitoneally or intramuscularly were approximately half as effective as pyridoxine in the feed in counteracting the growth inhibitor.

Pyridoxine, pyridoxal and pyridoxamine were equally effective, on a molar basis, in counteracting the growth inhibitor.

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ENZYMES IN PROTEIN DEPLETION

II. OXIDATIVE ENZYMES OF HEART VENTRICLE [1]

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TWO FIGURES

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Although many investigators have studied the effects of protein depletion on the succinic dehydrogenase and cytochrome oxidase activities of liver (Elson, '47; Lang, '47; Potter and Klug, '47; Benditt, Steffee, Hill and Johnston, '49; Millman, '51; Bargoni, '51; Wainio, Eichel, Eichel, Person, Estes and Allison, '53), there have been no previous studies with heart muscle. The present investigation is concerned with cytochrome oxidase, succinic dehydrogenase and DPN-cytochrome *c* reductase of heart ventricle and it demonstrates that the unit activities of these enzymes are unaffected by feeding a protein-free diet. Only the total activities which are related to the size of the heart are reduced.

EXPERIMENTAL

The male albino rats used in these two experiments were obtained from the Wistar Institute. They weighed approxi-

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mately 250 gm when received and they were treated in the manner previously described (Wainio et al., '53), except that in experiment 1 (rats 61 to 75) the 5 animals of each group were not fed individually. The animals given the protein-free diet (hereafter referred to as the depleted animals) were fed as a group, and the animals given the complete diet (hereafter referred to as the pair-fed animals) were pair-fed against the 5 depleted animals. The 5 animals receiving the complete diet ad libitum (hereafter referred to as the ad libitum animals) were also fed as a group. Therefore, in experiment 1 there was no record of individual food intakes. In experiment 2, however, the animals were kept in individual cages and one control was pair-fed against a depleted animal, while another control was fed ad libitum.

The complete diet contained 18% of casein and there was an isocaloric substitution of glucose for protein in the protein-free diet.

A daily record of food consumption and a weekly record of body weights was kept during the 7 weeks of each experiment.

At the end of 7 weeks, three rats (one out of each group) were sacrificed by decapitation on each of 5 successive days. The ventricles were weighed and a piece was homogenized with three volumes of cold 0.9% KCl in a Ten Broeck glass tissue homogenizer. The 1 : 20 homogenate was further diluted with the KCl to a final dilution of 1 : 280.

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

The methods for succinic dehydrogenase and DPN-cytochrome *c* reductase are presented in the first paper (Wainio et al., '53). Cytochrome oxidase was determined by a spectrophotometric method involving the oxidation of ferrocytochrome *c:* 2 ml of a solution made by mixing equal parts of a solution of cytochrome *c* (1 mg per ml) and of 0.1 M phosphate buffer pH 7.4, reduced with sodium hydrosulfite and

aerated, was pipetted into the cuvette; 0.9 ml of water was added and 0.1 ml of a 1:280 dilution of the heart homogenate. The temperature was 25°C.

RESULTS

In experiment 1 (rats 61 to 75) the average total food intake of the ad libitum animals amounted to 1,700 gm at the end of 7 weeks, while the depleted and pair-fed animals consumed only 970 gm. The intakes in experiment 2 (rats 81 to 95) were 1,801 and 1,163 gm, respectively.

TABLE 1

Weight and total protein of heart ventricles

EXPERIMENT	GROUP	RAT NOS.	AVERAGE TOTAL WEIGHT	AVERAGE TOTAL PROTEIN
			gm	*gm*
	Depleted	61–65	0.498	0.093
			(0.441–0.580)	(0.075–0.112)
1	Pair-fed	66–70	0.644	0.138
			(0.577–0.805)	(0.118–0.184)
	Ad libitum	71–75	0.882	0.187
			(0.787–0.977)	(0.171–0.227)
	Depleted	81–85	0.549	0.101
			(0.506–0.576)	(0.097–0.109)
2	Pair-fed	86–90	0.824	0.157
			(0.755–0.873)	(0.148–0.163)
	Ad libitum	91–95	0.860	0.160
			(0.763–0.906)	(0.141–0.171)

The depleted rats in experiment 1 had an average body weight of 162 gm at the end of 7 weeks. The pair-fed animals weighed 263 gm and the ad libitum animals weighed 376 gm. In experiment 2 the weights were 171, 337 and 355 gm, respectively.

The ventricle weights and their protein contents are presented in table 1. The total protein is approximately 18.7% of the total weight of the heart in 4 of the 6 groups. The animals 66 to 70 and 71 to 75 average 21.4 and 21.2%, respectively. There is no ready explanation for this difference.

TABLE 2

Enzyme activities of heart ventricles

ENZYME	GROUP	RAT NOS.	AVERAGE UNIT ACTIVITY	AVERAGE TOTAL ACTIVITY	RAT NOS.	AVERAGE UNIT ACTIVITY	AVERAGE TOTAL ACTIVITY
Cytochrome oxidase [1]	Depleted	61–65	2.42 (1.64–3.03)	35.4 (25.2–50.3)	82–85 [3]	4.36 (4.13–4.56)	71.1 (64.1–74.5)
	Pair-fed	66–70	2.67 (2.40–3.05)	56.0 (46.8–68.7)	86–90	4.67 (3.79–5.64)	117.2 (97.0–147.0)
	Ad libitum	71–75	2.48 (1.57–3.70)	74.7 (45.7–100.8)	91–95	5.11 (4.44–5.98)	130.4 (118.5–163.8)
Succinic dehydrogenase [1]	Depleted	61–65	0.433 (0.355–0.527)	6.39 (4.68–7.38)	81–85	0.365 (0.285–0.440)	5.77 (4.62–6.62)
	Pair-fed	66–70	0.449 (0.323–0.592)	10.02 (6.62–13.30)	86–90	0.433 (0.348–0.515)	10.98 (8.20–13.40)
	Ad libitum	71–75	0.499 (0.322–0.705)	14.79 (9.27–20.40)	91–95	0.452 (0.410–0.525)	11.44 (9.27–13.00)
DPN-cytochrome *c* reductase [2]	Depleted	61–65	3.66 (2.61–4.56)	50.6 (41.5–64.5)	81–85	5.00 (4.53–5.97)	79.2 (73.0–87.5)
	Pair-fed	66–70	3.58 (3.03–4.13)	75.2 (59.0–87.0)	86–90	5.95 (5.39–6.55)	149.7 (127.0–170.5)
	Ad libitum	71–75	4.22 (3.42–4.88)	125.6 (98.4–138.4)	91–95	6.06 (5.57–6.89)	154.9 (129.3–173.5)

[1] Activities are expressed as 1st order velocity constants (sec^{-1}) per milligram N (unit activity) and per ventricle (total activity).
[2] Activities are expressed as zero order velocity constants (moles ferricytochrome *c* reduced per sec.) per milligram N (unit activity) and per ventricle (total activity).
[3] There were only 4 animals in this group.

The results of the cytochrome oxidase assays are presented in table 2. In experiment 1 (rats 61 to 75) the average unit activities, expressed as the first order reaction velocity constants, are not significantly different for the three groups. In experiment 2 the average unit activity for the ad libitum animals might possibly be significantly higher than for the depleted animals.

The higher unit activities for cytochrome oxidase in experiment 2 when compared to the unit activities in experiment 1 are difficult to explain. There is at least one possibility: that the difference may be due to the cytochrome *c* used in the assays. In experiment 1 the cytochrome *c* used was very generously supplied by Mr. Gerlough of the Squibb Institute for Medical Research. The reactions were between first and second order with respect to cytochrome *c*. In the second experiment (rats 81 to 95) cytochrome *c* from Wyeth, Inc., was used. In this instance the reactions were all first order. The velocity constants were calculated as first order reactions by fitting the best straight line to the points on a plot of the log of the cytochrome *c* concentration vs. time.

The total activities for cytochrome oxidase parallel the total heart proteins. The ad libitum animals have the greatest average total activity and the most protein in the heart. The depleted animals have the smallest average total activity and the least amount of protein in the heart. The pair-fed animals are intermediate in both respects.

The succinic dehydrogenase assays are also presented in table 2. Since the unit activities are not significantly different the total activities are proportional to the heart proteins. The results for the two experiments agree very well.

The DPN-cytochrome *c* reductase assays are also presented in table 2. The average unit activities show a tendency to be higher in the ad libitum animals, but the differences are probably not significant. The total activities are proportional to the heart proteins.

DISCUSSION

A comparison of these results with those previously obtained in liver reveals some differences. In figure 1 are presented the total protein and the total enzyme activity (per organ) in the depleted rats expressed as per cent of pair-fed

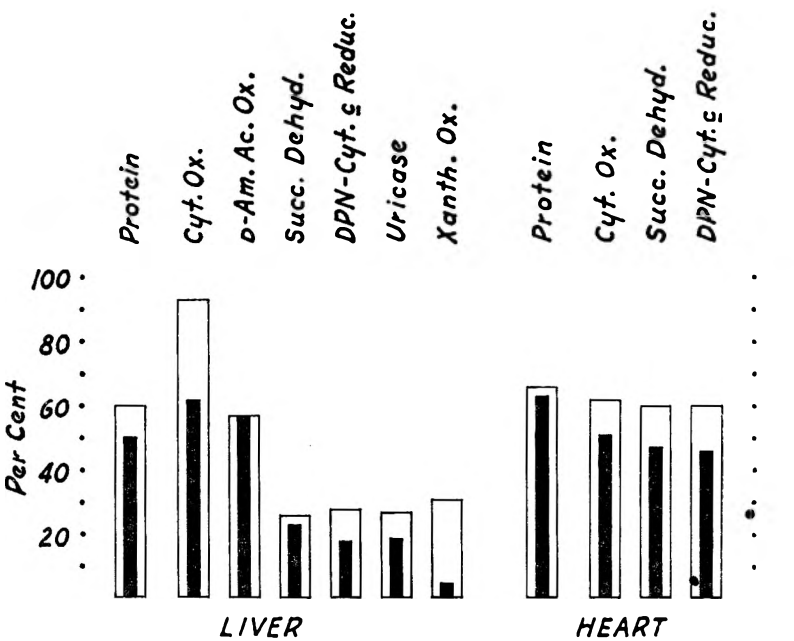

Fig. 1 Total protein and total enzyme activity in protein-depleted rats expressed as per cent of pair-fed controls (outer white bar) and as per cent of ad libitum fed controls (inner black bar). Cyt. Ox. = cytochrome oxidase, Succ. Dehyd. = succinic dehydrogenase, D-Am. Ac. Ox. = D-amino acid oxidase, DPN-Cyt. *c* Reduc. = DPN-cytochrome *c* reductase, Xanth. Ox. = xanthine oxidase, The data for liver are from the first paper of this series (Wainio et al., '53).

controls and as per cent of ad libitum fed controls. When comparing the depleted rats with their pair-fed controls it is apparent that cytochrome oxidase of liver is not markedly affected by the removal of the protein from the diet, whereas succinic dehydrogenase, DPN-cytochrome *c* reductase, uricase and especially xanthine oxidase are reduced much more than is the total protein. In contrast, the three enzymes of heart

are reduced approximately to the same extent as is the protein. A comparison of the activities in the depleted rats and the ad libitum fed rats emphasizes these differences, except that cytochrome oxidase in liver is now reduced and roughly

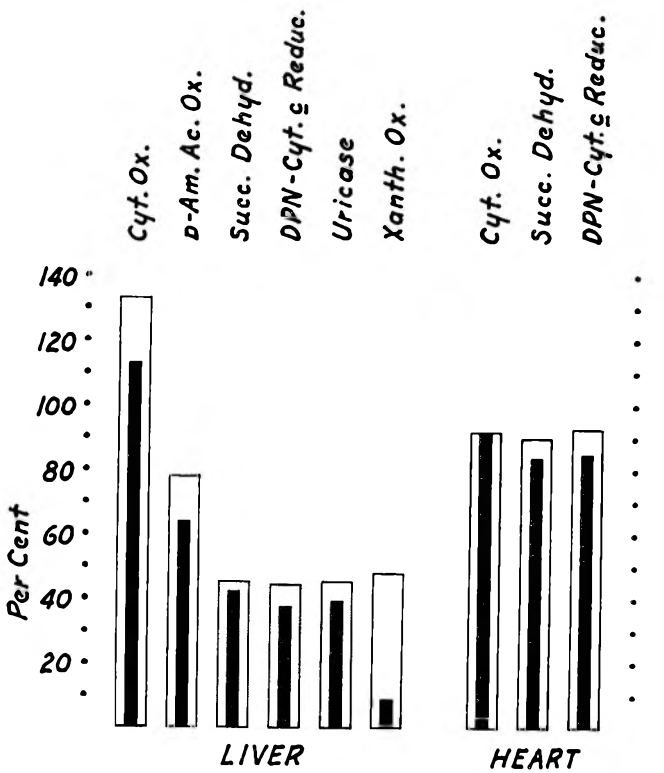

Fig. 2 Unit enzyme activity (per mg nitrogen) in protein-depleted rats expressed as per cent of pair-fed controls (outer white bar) and as per cent of ad libitum fed controls (inner black bar). Cyt. Ox. = cytochrome oxidase, Succ. Dehyd. = succinic dehydrogenase, D-Am. Ac. Ox. = D-amino acid oxidase, DPN-Cyt. *c* Reduc. = DPN-cytochrome *c* reductase, Xanth. Ox. = xanthine oxidase. The data for liver are from the first paper of this series (Wainio et al., '53).

parallels the reduction in protein, and that the enzymes of heart are reduced slightly more than is the protein.

A comparison of the unit activities (per mg nitrogen) in the depleted rats with the activities in the pair-fed and the ad libitum fed controls (fig. 2) reveals that cytochrome oxi-

dase of liver is conserved in the face of the loss of other enzymes, while xanthine oxidase is markedly reduced. In contrast, the enzymes of heart maintain their normal relationship to the nitrogen.

These differences in the responses of the heart and the liver may reflect the roles that each enzyme plays in the functioning of the organ, the role that each organ plays in the physiology of the animal, or both. The enzymes of liver are susceptible to different degrees, cytochrome oxidase being most stable and xanthine oxidase being most labile. The differences may reflect the manifold functions of the liver. On the other hand, the three enzymes of heart are all resistant and are reduced only as is the total nitrogen (or protein). These changes may reflect the single role of the heart in the organism.

SUMMARY

The cytochrome oxidase, succinic dehydrogenase and DPN-cytochrome *c* reductase activities of rat heart ventricle have been assayed after the animals were fed a protein-free diet for 49 days. The accompanying sub-acute food restriction, amounting to 41 and 35%, respectively, in two experiments, was controlled with pair-fed animals which received a diet containing 18% of casein. Ad libitum fed animals which were also given the complete diet served as further controls.

Neither protein depletion with its attendant food restriction nor food restriction alone had any significant effect on the unit activities of these three enzymes. The total activities of all of these enzymes were roughly proportional to the total proteins of the heart. The animals fed the basal diet ad libitum had the highest total activity and the largest amount of total protein, whereas the animals fed the protein-free diet had the lowest total activity and the least amount of total protein. The pair-fed controls had an intermediate total enzyme activity and an intermediate amount of protein in the heart.

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COMPOSITION OF INTESTINAL LUMEN LIPIDES FOLLOWING THE FEEDING OF TRIGLYCERIDES, PARTIAL GLYCERIDES OR FREE FATTY ACIDS [1]

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The isolation and identification of the intermediates formed by the enzymatic hydrolysis of triglycerides in the intestinal tract were recently reported from this laboratory (Mattson et al., '52). This hydrolysis, rather than being a random reaction, appears to be substantially a directed one from triglyceride to 1, 2-diglyceride to 2-monoglyceride. Subsequent isomerization of 2-monoglyceride to 1-monoglyceride also occurs in the intestinal tract. The establishment of this series of reactions still left unanswered the questions of further change in the monoglyceride and the form in which absorption took place.

This earlier work was limited to glycerides consisting of long-chain fatty acids. There was the possibility that the same types of intermediates would not accumulate during the hydrolysis of glycerides of short-chain fatty acids In addition, the use of monoglycerides and diglycerides as edible materials made it of interest to investigate the effect, if any, of these partial glycerides on the process of fat digestion. It was hoped that such studies would yield further information on the mechanisms of digestion and absorption of triglycerides.

EXPERIMENTAL

The experimental procedure used in this work was similar to that employed in the earlier experiments (Mattson et al., '52).

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Groups of three rats each that had been fasted for 48 hours were administered the test material by stomach tube. After a 3-hour interval the animals were sacrificed and the contents of the intestines were recovered and pooled by groups. These

TABLE 1

Digestion products of various lipides[1]

LIPIDE FED	DG + TG	TOTAL MG	1-MG	2-MG	FREE FA
Series I					
CSO	60.6	11.1	7.7	3.4	28.3
CSO	67.3	8.2	3.2	5.0	24.5
CSO	62.8	11.6	7.5	4.1	25.6
Series II					
Trieaprin	72.7	10.9	4.0	6.9	16.4
Series III					
92 CSO + 8 1-MG	61.8	12.2	6.8	5.4	26.0
79 CSO + 21 1-MG	66.6	11.9	6.8	5.1	21.5
Series IV					
92 CSO + 8 2-MG	72.5	8.5	1.9	6.6	19.0
89 CSO + 11 2-MG	68.4	10.6	4.2	6.4	21.0
74 CSO + 26 2-MG	68.8	11.7	4.1	7.6	19.5
70 CSO + 30 2-MG	59.2	15.8	4.3	11.5	25.0
Series V					
95 CSO + 5 FA	59.4	14.7	14.7	0	25.9
85 CSO + 15 FA	64.0	10.4	10.4	0	25.6
73 CSO + 27 FA	54.9	9.8	7.8	2.0	35.3
50 CSO + 50 FA	47.0	5.6	5.6	0	47.4

[1] All values represent percentage composition by weight. The following abbreviations are used: TG, triglyceride; DG, diglyceride; MG, monoglyceride; FA, fatty acid; CSO, cottonseed oil.

were analyzed for 1-monoglyceride content by the method of Pohle and Mehlenbacher ('50). Total monoglyceride was determined by the same method after conversion of 2-monoglyceride to 1-monoglyceride by treatment with perchloric acid (Martin, '53). The difference between the two values is then the quantity of 2-monoglyceride initially present. Free fatty

acids were determined by titration. All values reported are percentage composition by weight.

The cottonseed oil used in these studies had been refined, bleached and deodorized. The tricaprin was prepared by direct esterification of glycerol with capric acid. The 2-monoglyceride used was 2-monopalmitin prepared by the method of Bergmann and Carter ('30). The 1-monoglyceride was isolated by molecular distillation from superglycerinated, partially hydrogenated cottonseed oil (iodine value = 80). The free fatty acids were prepared by hydrolysis of cottonseed oil. All of these materials were of better than 90% purity.

RESULTS AND DISCUSSION

In the first series of experiments, three groups of rats were administered cottonseed oil, whose triglycerides are made up predominantly of fatty acids having a chain length of 18 carbon atoms. In series II a group of animals received tricaprin. In series III, IV and V, mixtures of cottonseed oil and 1-monoglyceride, 2-monoglyceride, or free fatty acid were administered to groups of animals. The analytical values obtained on the pooled lipides recovered from the lumen of the intestines of the various groups are shown in table 1.

The values obtained in series I indicate the variation that is found between various groups of rats when a triglyceride is fed. The relative proportion of 1- and 2-monoglyceride probably depends on the amount of isomerization taking place in the intestinal tract as a normal physiological process and that occurring during the processing of the lipides.

In series II, where tricaprin was fed, the composition of the lipides of the lumen in terms of relative amounts of complete and partial glycerides was similar to that when cottonseed oil was fed. Recent experiments by Kiyasu et al. ('52) have demonstrated that long-chain fatty acids are absorbed by way of the lymphatic system, whereas capric and other short-chain fatty acids are predominantly absorbed by way of the portal venous system. It is of interest that glycerides of both types of fatty acids yield similar digestion products. Quantitatively

the only difference appears to be a more rapid rate of disappearance of the shorter-chain free fatty acids.

The striking feature in the results obtained in series III, where mixtures of cottonseed oil and 1-monoglyceride were administered, is the remarkable constancy of composition of the lipides of the lumen of the intestinal tract, regardless of the mixture of complete or partial glycerides fed. The proportion of triglyceride and 1-monoglyceride fed varied from pure triglyceride (series I) to a 79-to-21 mixture of triglyceride and 1-monoglyceride (series III). Yet this resulted in no significant change in the composition of the lipides present in the lumen of the intestine.

In the next series, experimental animals were administered mixtures in varying proportions of triglyceride and 2-monoglyceride. The results obtained were similar to those seen when 1-monoglyceride was fed. Thus, when even relatively large amounts of 2-monoglyceride were fed, there was only a slight change in the composition of the lipides of the intestinal lumen. Only at the highest level at which 2-monoglyceride was administered, 30% of the fat, was there a significant change in the composition of the intestinal lumen lipides.

The results obtained where the partial glycerides were fed show that the presence of as much as 20% monoglyceride in dietary fat will not influence the composition of the lipides found in the lumen of the intestine. Studies at much higher dietary monoglyceride levels would be of little value because of the marked change in the physical properties of the fat.

In the final series of experiments various proportions of triglyceride and free fatty acid were fed. It will be noted that in these experiments there was almost a complete absence of 2-monoglyceride in the lipides recovered from the lumen of intestinal tract. Besides this absence of 2-monoglyceride, there were higher levels of free fatty acids in the lumen when the fed lipide contained 27% or more of free fatty acid. This would indicate that at these higher levels the rate of administration of free fatty acid was in excess of the rate of absorp-

tion. Borgstrom ('52) has made similar observations with respect to free fatty acid accumulation in the intestine.

The disposition of the dietary 1- or 2-monoglyceride or free fatty acid in series III, IV and V is not apparent at this time. Borgstrom ('52) has reported that following the feeding of triglyceride and free fatty acid there is a 28% randomization among the fatty acids and glycerides. On the other hand, we have fed rats mixtures of 1-monoglyceride and free fatty acid and have been unable to find 2-monoglyceride, diglyceride or triglyceride in the lumen of the intestine. Reiser et al. ('52) have reported that the main bulk of administered triglyceride is hydrolyzed to monoglyceride and absorbed in this form. If monoglycerides are absorbed as such, it would explain the relatively small rise in the monoglyceride content of the intestinal tract when large amounts of this partial glyceride are fed. At this time there are insufficient data on the role of esterification reactions in the digestive process to allow any conclusions as to the reactions undergone by the partial glycerides or free fatty acids fed in these experiments.

SUMMARY

1. The quantity and isomeric form of the monoglycerides found in the lumen of the intestinal tract during digestion are similar, regardless of whether the triglyceride fed consists of fatty acids having a chain length of 18 carbon atoms or one of 10 carbon atoms.

2. The composition of intestinal lipides is only very slightly affected when relatively large amounts of 1- or 2-monoglyceride are fed in admixture with triglyceride.

3. When a mixture of fatty acid and triglyceride is fed, the composition of the intestinal contents, except for relatively smaller amounts of 2-monoglyceride, is unchanged at low levels of fatty acid feeding but exhibits a marked rise in free fatty acid content when higher levels of free fatty acids are administered.

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THE BIOLOGICAL VALUE OF ALASKA PEA PROTEINS[1]

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Esselbaugh et al. ('52), using the egg replacement method with adult human subjects, reported that Alaska pea proteins were 95% as efficiently utilized as egg proteins. Supplementation of pea diets with methionine increased the egg replacement value of pea proteins to 100% or better for all subjects. This work indicated that the Alaska pea was a protein food of high biological value in human diets. There is evidence, however (Mitchell and Hamilton, '29; Sumner and Murlin, '38), that the biological values of foods are not the same for different species.

During processing, the most important agent modifying the nutritive value of the proteins of foods is heat, which usually depresses their nutritive qualities. This is well illustrated by the work of Morgan and King ('26), Stewart et al. ('43), and Mitchell and Block ('46). Woods et al. ('43), using growing rats as experimental animals, reported that heat treatment impaired the protein efficiency of Alaska peas. Murray ('47) re-

[1] Scientific Paper no. 1249, Washington Agricultural Experiment Stations, Pullman. Project no. 1091.

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ported a detrimental effect of heat upon pea proteins, probably because the cystine content was lowered.

Woods et al. ('43) showed that the lack of available methionine was the principal growth-limiting deficiency of raw Alaska field peas. They also showed that the most efficient level of supplementary methionine for a 10% raw pea-protein ration was not more than 0.3% of the diet.

Mitchell and Beadles ('50) found that the digestibility of proteins does not differ greatly for growing rats, adult rats, and adult human subjects but that the biological values are quite different for those proteins deficient in cystine-methionine. They compared their results with those obtained by Hawley et al. ('48), who had conducted a similar experiment with human subjects and concluded that the cystine-methionine requirement of the adult rat is more intense in relation to requirements for other amino acids than that of the growing rat or of the adult human being.

It was with the above findings and viewpoints in mind that the experiments reported herein were undertaken. The purpose of these experiments was to determine the biological value of raw and cooked Alaska pea proteins with and without methionine supplementation, using weanling and adult rats.

EXPERIMENTAL

Experiment 1

The Mitchell method ('24) was used in the first experiment to determine the biological value of raw and cooked Alaska pea proteins with and without methionine supplementation. The experimental animals consisted of 6 weanling (3 male and 3 female) albino rats of the same litter. The composition of experimental rations is presented in table 1.

The semi-purified rations were of 4 types: (1) raw peas alone, (2) raw peas plus 0.3% methionine, (3) cooked peas alone, and (4) cooked peas plus 0.3% methionine. Peas or peas plus methionine supplied the sole source of protein and all test rations were equalized to contain 9% protein, 7% fat,

TABLE 1

Composition of experimental rations

INGREDIENT	RATIONS (EXPERIMENT 1)					RATIONS (EXPERIMENT 2)				
	1	2	3	4	5	1	2	3	4	5
	Percentage					*Percentage*				
Dried whole egg	....	....	....	....	9.00	....	....	....	....	13.50
Raw peas	39.60	38.70	....	....	...	26.40	25.52	....	....	....
Cooked peas	....	....	35.89	35.09	...	....	....	23.92	23.13	....
Sucrose	42.23	42.76	45.64	46.08	73.56	48.08	48.55	50.32	50.75	64.68
Corn oil	6.60	6.61	6.64	6.65	3.10	13.07	13.08	13.09	13.10	7.48
Non-nutritive fiber (solka floc)	7.23	7.29	7.49	7.54	10.00	8.15	8.21	8.33	8.38	10.00
Salt mixture [1]	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
DL-methionine	....	0.30	...	0.30	...	...	0.30	...	0.30	...
Vitamin supplement [2]	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34

[1] Wesson, L. G. ('32).

[2] To each 100 gm of feed the following amounts of vitamins were added: choline, 300 mg; para amino benzoic acid, 30 mg; pantothenic acid, 2 mg; niacin, 1 mg; thiamine, 0.3 mg; riboflavin, 0.3 mg; pyridoxine, 0.3 mg; menadione, 0.1 mg; vitamin B_{12}, 2 μg and folic acid, 2 μg. Each animal also received 2 drops of a vitamin A and D feeding oil containing 100 I.U. of vitamin A and 40 I.U. of vitamin D and 25 mg of mixed tocopherols weekly.

and 10% fiber. The peas designated as cooked were soaked overnight with one and one-half cups of distilled water to 133 gm of peas. They were then placed in an oven and cooked at 250°F. for two and one-half hours in a covered casserole. No water was poured off during the procedure. All peas used in the experiment were dehydrated and fed in a finely ground form . The peas were from the same lot used by Esselbaugh et al. ('52).

For estimation of metabolic fecal nitrogen and endogenous urinary nitrogen excretions by each of the experimental animals, a semi-purified 4% protein ration (table 1, experiment 1, ration 5) was fed preceding and following the periods when the test rations were fed (Mitchell and Carmen, '26).

A balanced incomplete block type of feeding design was employed. Metabolic periods were 8 days in length and feces and urine collections were made for the last 5 days in each period. A 7-day adjustment period was allowed preceding the first low nitrogen period. Two per cent ferric oxide was included in the ration for the first feeding of each collection period and the first feeding followed the collection periods (Schneider, '35). By this means, feces were marked and only the feces resulting from the diet fed during the actual metabolic period were collected for analysis. The animals were kept in individual metabolism cages. Pyrex plates containing close-fitting, acidified filter paper were used for collection of urine and feces. Urine was extracted daily from the filter paper by washing with warm, acidified distilled water and the washings were kept in stoppered bottles. The urine washings were pooled for each collection period for each rat and made up to a standard volume. Aliquots were taken and nitrogen determinations were made in duplicate by the macro-Kjeldahl method. The feces were collected daily and kept in stoppered Kjeldahl flasks containing concentrated sulfuric acid. The entire collection for each animal was digested at the end of each period, transferred quantitatively to a volumetric flask, cooled, made up to a standard volume, and aliquots taken for Kjeldahl distillation and nitrogen determination.

Body weights of the rats were taken at the beginning and end of each metabolic period and their average weights determined. Accurate records were kept of the daily feed consumption for each animal. Endogenous urinary nitrogen excretions were estimated as the 0.75 power of body weight, and metabolic nitrogen of the feces was estimated on the basis of milligrams of nitrogen excreted per gram of dry matter consumed (Mitchell, '24).

Experiment 2

An effort was made in this experiment to approximate conditions maintained in the human experiment conducted by Esselbaugh et al. ('52). The egg replacement method (Murlin and Mattill, '38) was used to determine the relative value of the proteins of raw and cooked Alaska peas with and without added DL-methionine.

The experimental animals consisted of 5 young adult female rats from the same litter. At the beginning of the experiment the rats ranged in weight from 229 to 264 gm. They had been fed a nutritionally adequate stock ration before being placed on experiment.

The semi-purified experimental rations were of 5 types: (1) raw peas alone, (2) raw peas plus 0.3% DL-methionine, (3) cooked peas alone, (4) cooked peas plus 0.3% DL-methionine, and (5) the egg diet. The composition of these rations is presented in table 1. All rations were equalized to contain 6% protein, 10% fiber, and 13.3% fat. Peas, peas plus methionine, or egg provided the sole source of protein, and the rations were calculated to be approximately isocaloric. An average carbohydrate-fat ratio of 4.8:1 was maintained in all rations. By calculation, protein contributed approximately 6%, fat 30%, and carbohydrate 64% of the total daily caloric intake. The peas and dried egg were taken from the same lots as used in experiment 1, and cooking procedure was the same as in experiment 1.

The experimental design was the Latin square. Random selection was used in assigning rations to particular animals during the 5 metabolic periods.

The entire experimental series lasted 44 days. The metabolic periods, housing of animals, collection of excreta, and chemical methods were the same as described in experiment 1.

TABLE 2

Biological values of pea protein obtained in experiment 1

RAT NO. AND SEX	RAW PEA DIETS		COOKED PEA DIETS		AVERAGE
	Unsupplemented	Plus methionine	Unsupplemented	Plus methionine	
1 ♂	66.3	80.3	57.6	76.5	70.2
2 ♀	64.8	75.1	63.6	77.4	70.2
3 ♂	58.5	84.2	62.9	82.1	71.9
4 ♀	51.0	76.6	50.5	69.5	61.9
5 ♂	57.7	71.1	65.0	74.7	67.1
6 ♀	65.0	78.0	70.5	81.0	73.6
Average	60.6	77.6	61.7	76.9	

TABLE 3

Body weight gains of animals in experiment 1

RATION	AVERAGE DAILY GAIN
	gm
Raw pea proteins alone	1.17
Cooked pea proteins alone	0.93
Raw pea proteins plus DL-methionine	2.50
Cooked pea proteins plus DL-methionine	2.33
Raw pea proteins (with and without DL-methionine)	1.83
Cooked pea proteins (with and without DL-methionine)	1.63
Raw and cooked pea proteins (no DL-methionine)	1.05
Raw and cooked pea proteins (plus DL-methionine)	2.42

RESULTS AND DISCUSSION

Experiment 1

Table 2 shows the average biological values obtained for each animal on the 4 dietary regimens.

The average biological values of unsupplemented raw and cooked Alaska pea proteins were found to be 60.6 and 61.7, respectively. The addition of 0.3% DL-methionine raised the

average biological value of raw pea proteins to 77.6 and that of cooked pea proteins to 76.9. Statistical analysis showed this increase in biological value of pea proteins due to methionine supplementation to be highly significant ($P < .01$). No significant difference in biological values was found between raw or cooked pea proteins or between rat sexes.

Apparent digestibility of the proteins in the 4 test rations was virtually the same, ranging from 78.1 to 79.4. The average true digestibility of the proteins of the test rations varied only from 86.2 to 87.2. Therefore, it appeared that cooking and

TABLE 4

Egg replacement values of pea proteins

RAT NO.	RAW PEAS DIETS		COOKED PEAS DIETS		AVERAGE
	Un-supplemented	Plus methionine	Un-supplemented	Plus methionine	
1	73.6	87.2	66.7	81.1	77.2
2	78.5	89.3	74.3	90.0	83.1
3	70.0	78.4	61.2	77.7	71.8
4	80.9	92.5	81.5	93.4	87.1
5	86.8	98.9	80.4	99.8	91.5
Average	78.0	89.3	72.8	88.4	

methionine supplementation had no effect on the digestibility of pea proteins.

Average daily weight gains of animals fed the various rations are presented in table 3.

The average daily gain of 2.42 gm by the animals when 0.3% DL-methionine was added to peas as compared with 1.05 gm daily gain with no supplementation supports the work of Woods et al. ('43) in showing that, with the single exception of methionine, the Alaska field pea is an excellent source of the amino acids essential for rat growth.

Experiment 2

Every rat maintained its weight with slight gains throughout this experiment. Table 4 shows the average egg replace-

ment values obtained for individual animals on the 4 pea rations.

Unsupplemented raw pea proteins and cooked pea proteins were found to be 78.0% and 72.8% as efficiently utilized, respectively, as egg proteins. The egg replacement value of 72.8% obtained for cooked pea proteins contrasts with the corresponding value of 95.1% reported by Esselbaugh et al. ('52) for mature human subjects. With the positive nitrogen retention of all animals upon the addition of 0.3% DL-methionine to the pea rations, average egg replacement values were increased to 89.3% for raw pea proteins and to 88.4% for cooked pea proteins. This increase in the efficiency of utilization of pea proteins obtained by adding 0.3% DL-methionine to the rations was highly significant ($P < .01$). The average egg replacement value of 88.4% obtained for cooked pea proteins plus 0.3% DL-methionine contrast with the corresponding value of 110.9% reported by Esselbaugh et al. ('52) for mature human subjects. Therefore, it appears that adult rats are less efficient in utilization of Alaska pea proteins than adult human beings.

Statistical analysis showed that the efficiency of utilization of unsupplemented raw pea proteins (78.0%) was significantly greater ($P < .01$) than that of unsupplemented cooked pea proteins (72.8%). This is in agreement with the work of Murray ('47) who reported that cooking has a harmful effect on pea proteins, probably because the cystine content is lowered.

The difference between the effects of methionine supplementation in combination with raw pea proteins and cooked pea proteins was considerable. Statistical analysis showed this interaction to be significant ($P < .05$). Adding methionine to the pea rations produced a greater average increase in the egg replacement value of cooked pea proteins (15.6 units) than of raw pea proteins (11.3 units). The cooking of peas decreased their egg replacement value when they were fed without methionine supplementation but did not do so when methionine was added to the diet. This result was in contrast to the results obtained in experiment 1 in which it was found that

cooking the peas had no effect on the efficiency of utilization of pea proteins by weanling rats. It is possible that cooking caused a significant effect with the mature rats because they were receiving a diet with a lower percentage of protein. However, this contrasting result in the two experiments is in accord with the report of Mitchell and Beadles ('50) that the cystine-methionine requirement of the adult rat is more intense in relation to requirements for other amino acids than that of the growing rat.

Egg proteins were found to be more digestible than the pea proteins, whether cooked or uncooked, methionine supplemented or unsupplemented. The average apparent coefficient of digestibility for egg proteins was 81.8% and for pea proteins, 73.6%. Analysis of variance showed this difference to be highly significant ($P < .01$). The cooking process or the addition of 0.3% DL-methionine to the pea diets had no apparent effect on protein digestibility. Average digestion coefficients obtained on the proteins of test rations varied only from 72.7% to 74.7% These digestion coefficients were lower than corresponding values reported by Esselbaugh et al. ('52) for human subjects. Therefore, it appears that egg and pea proteins are less efficiently digested by rats than by human subjects.

SUMMARY

The average biological values of raw and cooked Alaska pea proteins fed at a 9% protein level as determined by the Mitchell method with weanling rats were found to be 60.6% and 61.7%, respectively. The addition of 0.3% DL-methionine to the diets increased the average biological value of raw pea proteins to 77.6% and that of cooked pea proteins to 76.9%. Average egg replacement values of raw and cooked Alaska pea proteins fed at a 6% protein level as determined by Murlin and Mattill's method with adult rats were found to be 78.0% and 72.8%, respectively. With the addition of 0.3% DL-methionine to the diets, average egg replacement values were increased to 89.3% for raw pea proteins and to 88.4% for cooked pea proteins. It was indicated that Alaska peas are a protein

food of high biological value when adequately supplemented by methionine.

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EFFECT OF POTASSIUM, SODIUM OR CALCIUM ON THE GROWTH OF YOUNG RABBITS FED PURIFIED DIETS CONTAINING DIFFERENT LEVELS OF FAT AND PROTEIN

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ONE FIGURE

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Wooley and Sebrell ('45) showed that rabbits weaned at 8 weeks of age grew at a nearly normal rate when fed a purified diet (diet 1395). When this diet was fed to three-week-old animals, the growth rate was poor (Wooley, '54a). The poor growth was improved when the diet was supplemented with kale or other vegetables and to a lesser extent by the addition of potassium (Wooley, '54a). Increasing the casein in the diet from 20 to 30% depressed growth and resulted in a high mortality. A similar phenomenon was noted when the 30% casein diet was fed to 8-week-old rabbits (Wooley, '54b). At first it was felt that the higher level of casein was toxic to rabbits. However, the good growth secured on another purified diet containing 30% casein (diet 1436, Wooley, '54a) indicated that under certain conditions rabbits could tolerate the higher level of protein in the diet. These two diets containing 30% casein differed, among other things, in the levels of minerals and fat.

The present studies were designed to determine the effect of adding extra amounts of calcium, potassium or sodium

to the purified diet which produced only poor growth (diet 1395). Variations in the fat and protein levels were also studied. By increasing the level of any one of the above minerals in the diets containing 30% casein, growth inhibition and mortality were overcome. The effect of minerals and fat supplements on the 30% casein diets was greater than on the 20% casein diets.

METHODS AND MATERIALS

Male rabbits from a white New Zealand strain reared at the National Institutes of Health by promiscuous breeding were used. The rabbits were weaned at three to 4 weeks of age, when they weighed 350 to 500 gm. At that time, they were placed in individual cages with wire screen bottoms. Littermates were distributed among the experimental groups. Each test involved several groups with 4 rabbits in each group. The animals were kept on the experiment for 4 weeks. Many experiments were repeated two or more times. Feed and water were available to the rabbits at all times. If any rabbit died during the first week of the experiment, data concerning it were not included in the final results; however, if an animal died at some later date, the data were included.

The control rabbits received pellets [1] (Wooley, '54a); the experimental animals received diets consisting of vitamin-free casein, 20 or 30%; cornstarch, 20%, cellophane spangles, 15%; the Wesson [2] modification of the Osborne and Mendel salt mixture, 4%; cottonseed oil, varying levels; vitamins used in diet 1395 (Wooley, '54a); and sucrose in amounts to make up to 100%. When extra minerals were added, equivalent amounts of sucrose were withheld. Potassium was added as acetate, sodium as bicarbonate, and calcium as carbonate. These minerals were used in amounts that brought the levles up to those in diet 1436 (Wooley, '54a).

[1] The pellets contained 17% protein, 55% nitrogen-free extract, 15% fiber and 3% fat.

[2] The Wesson salts supplied 0.6% potassium, 0.17% sodium and 0.57% calcium in the diet.

TABLE 1

Growth of rabbits fed stock pellets and a purified ration containing varying levels of casein, fat and minerals

(Average weights are for the rabbits alive at end of experiments)

PROTEIN	FAT	NO. RABBITS [2]	AVE. WT. GAIN IN 28 DAYS
%	%		gm
	Commercial rabbit pellets		
17	3	19	981 ± 27
	Purified diets (no extra minerals)		
20	1 [1]	11 (1)	393 ± 31
20	5	16 (1)	347 ± 39
20	8	8 (1)	307 ± 67
20	12	7 (1)	228 ± 36
30	1	4 (3)	120 [3]
30	5	11 (8)	260 [3]
30	8	12 (5)	107 [3]
30	12	8 (3)	382 [3]

CASEIN	MINERAL	NO. RABBITS	AVE. WT. GAIN IN 28 DAYS
%	%		gm
	Purified diets (mineral supplements)		
	Fat 1%		
20	0.8 K [4]	6	486 ± 60
20	0.4 Na [5]	7	521 ± 59
20	0.73 Ca [6]	7 (1)	428 ± 62
30	0.8 K	4 (1)	413 ± 103
30	0.4 Na	4 (1)	430 ± 66
30	0.73 Ca	4	520 ± 52
	Fat 5%		
20	0.8 K	11	465 ± 21
20	0.4 Na	12	596 ± 49
20	0.73 Ca	6	572 ± 48
30	0.8 K	7	590 ± 81
30	0.4 Na	6	742 ± 47
30	0.73 Ca	7 (1)	757 ± 47
	Fat 8%		
20	0.8 K	7	386 ± 60
20	0.4 Na	11	606 ± 32
20	0.73 Ca	7	470 ± 80
20	0.8 K, 0.4 Na and 0.73 Ca	4	675 ± 72
30	0.8 K	8 (3)	602 ± 46
30	0.4 Na	8	854 ± 54
30	0.73 Ca	5	664 ± 54
30	0.8 K, 0.4 Na and 0.73 Ca	3	727 ± 120
	Fat 12%		
20	0.8 K	8	522 ± 29
20	0.4 Na	11	618 ± 47
20	0.73 Ca	7	637 ± 66
30	0.8 K	6	785 ± 57
30	0.4 Na	8	905 ± 62
30	0.73 Ca	4	855 ± 61
30	0.8 K, 0.4 Na and 0.73 Ca	4	973 ± 52

[1] Cottonseed oil.

[2] Numbers in parentheses represent rabbits surviving and gaining weight for one week or longer and dying before termination of experiment.

[3] Standard error of the mean not determined due to high mortality.

[4] Potassium added as acetate.

[5] Sodium added as bicarbonate.

[6] Calcium added as carbonate.

RESULTS

Statistical analysis showed that variations in weight gained (for a given diet) from test to test were no greater than those seen among the same animals on the same diet in any one test. For this reason, the results from all rabbits fed the same diet were pooled.

Increasing the fat content of the diets from 1 to 12% in the absence of any extra minerals produced a decrease in the

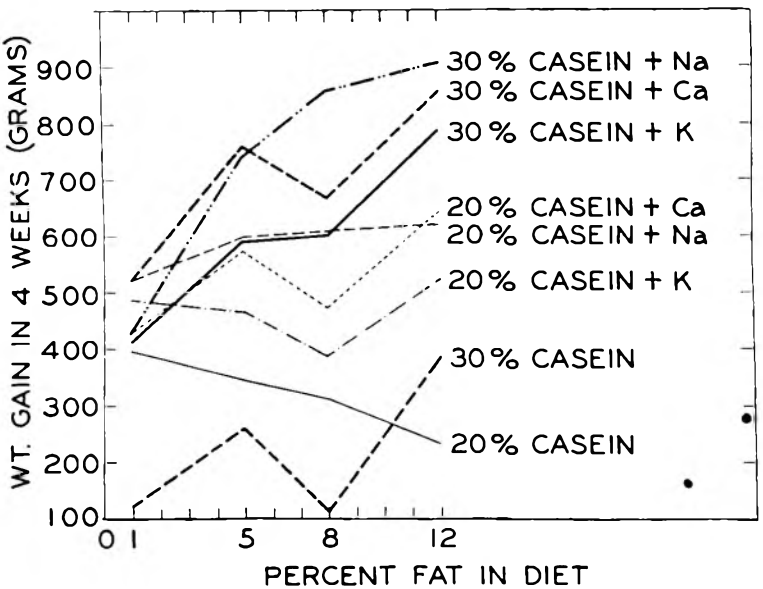

Fig. 1 Influence of fat, protein and cations on the growth of young rabbits.

growth of three- to 4-week-old rabbits on 20% casein (table 1 and fig. 1). The response of the rabbits to the 30% casein diets without any extra minerals was difficult to interpret because of the high mortality with all levels of fat (table 1). The high mortality was not seen when extra minerals were used in the diet.

There was no significant difference in the growth of the rabbits on the 1% fat diets containing 20 or 30% casein supplemented with extra minerals. When any one of the min-

erals was added to the diets containing 5% or more fat and 30% casein, the growth rates increased with increasing levels of fat in the diet. When diets containing 20% casein and extra minerals were used, the growth responses to increasing dietary fat levels were much less than on the 30% casein diets. The growth response of rabbits on the 8% fat diets deviated from that which would have been predicted from the growth responses with 1, 5 and 12% fat levels. These deviations occurred in several repeat experiments and made it impossible to establish a statistical correlation between growth and dietary fat. Supplementation of the diets with sodium or calcium appears to result in greater growth responses than when potassium is used.

When all three minerals were added to the diets containing either 20 or 30% casein and 8 or 12% fat, the growth response was not significantly different from that obtained with sodium (table 1). It might be noted that on the 12% fat diet the addition of either sodium alone or all three minerals produced growth comparable to that obtained with commercial rabbit pellets.

DISCUSSION

It is difficult to rationalize the marked improvement in growth of the rabbits which occurred when either sodium, potassium or calcium was added to the experimental ration. The levels of these minerals and of casein in the basal diet were comparable to those in the purified diets which promote good growth in rats.

The addition of all three minerals to the diet produced no greater growth than that secured when only sodium was used. The significance of this observation is, to say the least, obscure. It might mean that the basal diet was deficient primarily in one mineral and that the addition of either one of the others spared the requirement for that substance. The presently available evidence (see below) would argue against this possibility.

Besides the minerals, the levels of casein and fat in the diet influenced the results. One of the basal purified diets

used in these studies contained 30% casein and 1% fat. The growth secured on this basal diet even when supplemented with extra minerals was significantly less than that obtained with rabbit pellets (table 1). The latter contained 17.0% protein, all of which was of plant origin, and 3% fat. The above observation suggests the presence in plant material of an unknown growth substance or a balance of nutrients that is more favorable for the rabbit. The minerals in plants have been partially ruled out as the unknown growth factor, since the ash from kale, when added to the basal diet, produced a growth stimulation no greater than that secured from potassium (Wooley, '54b). Growth comparable to that seen with pellets (over a 4-week period) can be secured with the purified diet if the fat content is increased to 12% and either calcium or sodium added (table 1).

Relatively little work has been done on the influence of dietary fat upon the growth of rabbits. Weanling rabbits have been reported to grow "normally" on powdered whole milk which contains 25% fat (Smith and Ellis, '47). It is not possible to evaluate the influence of varying dietary fat levels on the growth of rabbits on the basis of this report. Furthermore, the weight gains of their rabbits over a 20-week period were no greater than those made by our rabbits in the present study during 4 weeks.

There is no information on the protein requirement of three-week-old rabbits and nothing specifically on the relation between that and minerals. A few reports have, however, appeared with respect to such a relationship for rats. Cannon, Frazier and Hughes ('52) found that protein-depleted rats failed to utilize a fibrin hydrolysate unless potassium was present in the diet. Results similar to these were secured by Frost and Sandy ('53), who also observed that the level of potassium necessary for an increase in body weight on the fibrin hydrolysate diet was the same as the suggested daily requirement under normal dietary conditions.

The response of protein-depleted rats to potassium deprivation is in marked contrast to that reported for normal rats

raised on a potassium-free diet. Orent-Keiles and McCollum ('41) stated that over a period of 9 weeks the retention of nitrogen by their rats on a potassium-free diet was the same as that of supplemented animals. It is difficult to reconcile the results of their balance studies with their growth curves, since the deficient rats gained approximately 65 gm and the supplemented rats 105 gm during the balance study. Further work will be necessary in order to clarify this point.

There is very little information on the dietary interrelationships of sodium and potassium. A review of studies on the relation of minerals to protein metabolism makes no reference to any work in that area (Abbott, '48). A recent paper by Cannon, Frazier and Hughes ('53) reviews the literature in this field and it also contains no references to work on the dietary interrelationships of these minerals. These investigators found that protein-depleted rats show a more acute and severe form of deficiency as the sodium content of their potassium-free diet is increased. Conversely, by decreasing the amount of sodium in their potassium-free diet, the heart and kidney lesions could be prevented even in prolonged experiments.

It should be noted that the work of Cannon et al. ('53) and Frost and Sandy ('53) was done with protein-depleted rats and thus differs markedly from the work reported in the present paper. All of our rabbits were on a normal diet at the start of the experiments. This fact plus the difference in species may explain the ability of sodium and calcium to replace potassium as supplements to a ration which, on a theoretical basis, would appear to be complete without supplementation. The complementary action of calcium and potassium under the circumstances of our tests is contrary to the behavior of these two minerals when perfused through the heart (Best and Taylor, '50). These findings are also at variance with the accepted antagonism between sodium and potassium as far as their concentration in cells and extracellular fluid is concerned (Cannon et al., '53).

There is a suggestion from the work of Olcese and Pearson ('48) that proteins may be important in fat metabolism. They found that rabbits on a 10% casein diet developed signs of a vitamin A deficiency, whereas no deficiency was apparent on a 20% casein diet which contained the same concentration of vitamin A. It was suggested that "since it is doubtful that the lower protein intake interferes with the absorption of fat-soluble factors, the mechanism by which the additional protein produces its beneficial effects is probably related to the synergistic effect of the amino acids in the utilization of the fat."

The older literature contains a few reports which may have a bearing on these findings. Weiske (1894) found that when a rabbit was fed nothing but oats, it lost weight over a three-month period. The total mineral and calcium content of the bones was reduced. Addition of calcium carbonate to the diet produced a slight increase in body weight and in the mineral content of the bones. That the calcium supplement did not act primarily by overcoming a dietary deficiency of this mineral was shown by Morgen and Beger ('15), who found that sodium carbonate but not sodium chloride was more effective than calcium in overcoming the condition. They suggested that the sodium carbonate acted to increase the alkali reserve. Funk ('16) partially confirmed the observations of Morgen and Beger. However, Funk found marked individual differences among his rabbits — some of them lived and reproduced on the oats without any supplement. Similar work with guinea pigs showed that sodium bicarbonate hastened death when added to the oats.

Perdue and Phillips ('52) observed that rats on a low potassium diet showed a higher incidence and greater degree of myocardial necrosis and fibrosis and a greater dilation of renal tubules on a diet containing 20% corn oil than on one containing only 5%. Perdue and Phillips were unable to explain their results. It might not be amiss to suggest that here, as in our studies, there is evidence of a relation between fat metabolism and dietary potassium. In our studies, the

growth-promoting effect of added potassium could be duplicated by sodium or calcium.

SUMMARY

1. The addition of extra amounts of sodium bicarbonate, potassium acetate or calcium carbonate (over that in the 4% Wesson salt mixture) to a purified diet increased growth and decreased mortality in three- to 4-week-old rabbits.

2. The effect of either of the three mineral supplements was greater on diets containing 30% casein than on diets containing 20%.

3. Increasing fat in the diet tended to decrease the growth of rabbits on the 20% casein diets not supplemented with extra minerals. When the added minerals were present, the growth rate on the 30% casein diets increased as the fat level was raised from 1 to 12%; the increase on the 20% diets was considerably less than on the 30% diets.

4. With levels of fat ranging from 5 to 12% in the diet, there was an indication that sodium and calcium produced greater weight gains than potassium. The latter diets containing 30% casein produced weight gains comparable to those obtained on commercial pellets.

5. The addition of all three minerals to the diet produced no greater growth than sodium.

6. No explanation is available for these observations other than the suggestion that an animal's requirement for minerals may be more intimately related to the composition of the diet than has been thought heretofore.

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DETERMINATION OF THE METABOLIZABLE ENERGY OF ORGANIC NUTRIENTS FOR THE RAT

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In the course of a study of the biological values of a protein obtained with rats on diets containing different ratios of carbohydrate and fat calories, it was considered essential to feed these diets in amounts containing the same metabolizable energy. No information was found in the literature concerning the metabolizable energy of the three main classes of organic nutrients for the rat. The authors did not believe that the Atwater factors of 4 cal. per gram for protein and carbohydrate and 9 cal. per gram for fat should be applied. Aside from the error incurred in using caloric conversion factors strictly applicable to well-balanced diets in human nutrition made up of natural foods, to synthetic diets of varied composition, there remained the uncertainty of applying to the rat caloric factors secured with human subjects.

Hence, determinations of the metabolizable energy (or fuel value) of the organic nutrients of immediate interest to the main study were carried out by a method involving multiple regression analysis of gross energy (heats of combustion) of the diets, feces and urines secured with 12 adult rats when subsisting successively on three diets containing widely different proportions of organic nutrients. The results are considered worthy of publication, first, because no such information with reference to the laboratory rat is available in the literature; second, because the results secured should be of value

[1] Government of Hyderabad (India) fellow.

in designing other experiments in which isocaloric amounts of metabolizable energy are to be fed to experimental animals; and, third, because the method of analyzing the data may have wide application in solving other nutritional problems.

TABLE 1

Composition of the experimental diets

CONSTITUENTS	DIET 1	DIET 2	DIET 3
Casein (Labco, vitamin-free)	43.0	10.0	20.0
Corn starch	10.0	73.0	30.0
Lard	38.0	8.0	41.0
Wheat germ oil	0.5	0.5	0.5
Cod-liver oil	1.5	1.5	1.5
Minerals [1]	7.0	7.0	7.0
Vitamins [2]			
Total	100.0	100.0	100.0

[1] Minerals consisted of 4 gm Mineral Mixture 446 (Spector, '48), 1 gm sodium chloride and 2 gm barium sulfate.

[2] Vitamins were added per 100 gm diet as follows: calcium pantothenate, 2 mg; choline chloride, 400 mg; pyridoxine hydrochloride, riboflavin and thiamine hydrochloride, 0.25 mg each; nicotinic acid, 1 mg; *p*-aminobenzoic acid, 5 mg; and inositol, 10 mg.

MATERIALS AND METHODS

Diets

Three calorigenic nutrients, casein (Labco vitamin-free), a mixture of fats (mainly lard), and corn starch were used in the three experimental diets formulated in such a way that there was a large variation in the proportions existing among these three nutrients. These diets, called diet 1, diet 2 and diet 3, respectively, had equal amounts of vitamins and minerals in them and were balanced for maintenance. Two per cent of barium sulfate was included in the diets to serve as roughage. The composition of the diets is given in table 1.

Animals and experimental plan

Twelve Sprague-Dawley adult male rats were divided at random into three groups of 4 rats each. A 3×3 latin square

design in all replications was used in the plan of feeding. The rats were individually caged in a rat unit during the 10-day prefeeding periods and were transferred to glass-bottom metabolism cages during the 10-day collection periods, to enable separate and accurate collection of urine and feces. The daily caloric requirements of the rats (Q) were estimated from Brody's generalized equation, $Q = 70.4\,W_{kg}^{.734}$ (Brody and Proctor, '32) for basal energy, to which was added 25% for activity increment. The metabolizable energy values of the

TABLE 2

Analyses of the diets per 100 gm

	DIET 1	DIET 2	DIET 3
Gross energy (cal.)	642.12	419.28	624.16
Total nitrogen (gm)	6.1713	1.4831	2.8515
Ether extract (gm)	39.44	9.97	42.72
Dry matter (gm)	97.16	92.46	96.88

TABLE 3

Analyses of the nutrients tested

	CASEIN	STARCH	FATS
Dry matter, %	93.12	90.35	
Heat of combusion per gram of dry substance (cal.)	5.831	4.197	9.494

three diets were estimated using Atwater's 4-9-4 factors, and once daily the rats were offered weighed amounts of the diets according to the estimated caloric requirements. Each rat consumed a constant amount of food daily for at least 9 days of the adjustment period and 10 days of the collection period. There was practically no feed refusal by any rat during the entire experiment, except that during the third collection period two rats became sick and were removed from the experiment. The rats maintained, approximately, their initial body weights during the entire experiment.

All rats were kept in an air-conditioned room, the temperature of which was maintained at 28 ± 1°C. and the humidity at 43 ± 5%.

Chemical analyses

The diets were analyzed for gross energy by burning in an oxygen bomb calorimeter, for total nitrogen by the Kjeldahl method, using mercury as a catalyst and for dry matter and ether extract by the standard A.O.A.C. procedures. Gross

TABLE 4

The results of the first experimental period expressed on the 10-day basis

RAT NO.	DIET NO.	GROSS ENERGY OF DIET CONSUMED	PURE NUTRIENTS CONSUMED			ENERGY LOST IN URINE AND FECES	NITROGEN BALANCE	METABOLIZABLE ENERGY OF DIET CONSUMED (AFTER CORRECTING TO NITROGEN EQUILIBRIUM)[1]
			Casein	Starch	Fat			
		cal.	*gm.*	*gm.*	*gm.*	*cal.*	*gm.*	*cal.*
1	1	430.22	26.83	6.05	26.80	50.04	+ 0.1894	379
2	1	449.48	28.03	6.32	28.00	55.56	+ 0.3700	392
3	1	404.53	25.23	5.69	25.20	41.76	+ 0.1892	362
4	1	404.53	25.23	5.69	25.20	41.69	+ 0.2300	362
5	2	398.32	8.85	62.63	9.50	24.94	+ 0.2419	372
6	2	398.32	8.85	62.63	9.50	25.23	+ 0.0909	373
7	2	398.32	8.85	62.63	9.50	24.84	+ 0.1109	373
8	2	398.32	8.85	62.63	9.50	21.70	+ 0.2760	375
9	3	405.71	12.11	17.61	27.95	34.48	+ 0.2110	370
10	3	436.91	13.04	18.97	30.10	37.35	+ 0.0476	399
11	3	405.71	12.11	17.61	27.95	38.70	— 0.0222	367
12	3	405.71	12.11	17.61	27.95	34.11	+ 0.1753	371

[1] See text.

energy and dry matter were determined on casein and starch. The mixed fats were assumed to be moisture-free. The results are shown in table 2 and table 3, respectively

Feces and urine of each rat were collected separately every day and pooled for the collection period. Thus, 34 samples of urine and feces were obtained. Urines were preserved under toluene at 4°C. and feces were suspended in about 1 N hydrochloric acid and were separated from hair by sieving. Total nitrogen was determined on urine and feces samples by the

Kjeldahl method, and gross energy in the bomb calorimeter after drying at a low temperature.

THE EXPERIMENTAL RESULTS AND THEIR ANALYSIS

The results obtained in the three experimental periods are given in full in tables 4, 5 and 6 for the 10-day collections.

It is necessary to correct the urinary energy in a determination of the metabolizable energy of a diet for protein stored in or lost from the body during the period of observation, since

TABLE 5

The results of the second experimental period expressed on the 10-day basis

RAT NO.	DIET NO.	GROSS ENERGY OF DIET CONSUMED	PURE NUTRIENTS CONSUMED			ENERGY LOST IN URINE AND FECES	NITROGEN BALANCE	METABOLIZABLE ENERGY OF DIET CONSUMED (AFTER CORRECTING TO NITROGEN EQUILIBRIUM)
			Casein	Starch	Fat			
		cal.	*gm*	*gm*	*gm*	*cal.*	*gm*	*cal.*
1	2	398.32	8.85	62.63	9.50	18.43	+ 0.1547	379
2	2	398.32	8.85	62.63	9.50	23.60	+ 0.1107	374
3	2	398.32	8.85	62.63	9.50	19.90	+ 0.0038	378
4	2	398.32	8.85	62.63	9.50	24.21	+ 0.1078	373
5	3	405.71	12.11	17.61	27.95	35.93	— 0.1135	371
6	3	436.91	13.04	18.97	30.10	36.70	— 0.1211	401
7	3	405.71	12.11	17.61	27.95	33.83	— 0.0899	373
8	3	405.71	12.11	17.61	27.95	31.90	— 0.0978	374
9	1	430.22	26.83	6.05	26.80	45.99	+ 0.0202	384
10	1	449.48	28.03	6.32	28.00	44.80	+ 0.1716	404
11	1	417.38	26.03	5.87	26.00	39.52	+ 0.0662	378
12	1	404.34	25.21	5.69	25.19	42.28	+ 0.0864	362

protein stored has not been catabolized, while protein lost from the body originated in the tissues and not in the food simultaneously consumed. Such a correction can be made from the nitrogen balance. It has been customary in this situation to use Rubner's (1885) factor of 7.45 cal. per gram of nitrogen balance, the product to be subtracted from the urinary energy if the balance is negative and to be added if the balance is positive. However, this factor was obtained with dogs and its applicability to the rat is uncertain.

An experiment was, therefore, carried out on 7 adult male rats to determine the ratio of energy to nitrogen in the urine. The rats were offered daily 16 gm of a diet containing 94% water-extracted lean beef with mineral, vitamin and roughage supplements as in previous diets. After 12 days of adjustment, individual urines were collected for 5 days, and the nitrogen and gross energy contents were determined. The average ratio of energy to nitrogen was 6.29 ± 0.19 cal. per gram of urinary

TABLE 6

The results of the third experimental period expressed on the 10-day basis

RAT NO.	DIET NO.	GROSS ENERGY OF DIET CONSUMED	PURE NUTRIENTS CONSUMED			ENERGY LOST IN URINE AND FECES	NITROGEN BALANCE	METABOLIZABLE ENERGY OF DIET CONSUMED (AFTER CORRECTING TO NITROGEN EQUILIBRIUM)
			Casein	Starch	Fat			
		cal.	*gm*	*gm*	*gm*	*cal.*	*gm*	*cal.*
1	3	468.12	13.97	20.32	32.25	40.55	+ 0.0434	427
2	3	468.12	13.97	20.32	32.25	35.65	+ 0.1669	432
3	3	468.12	13.97	20.32	32.25	40.67	+ 0.1278	427
4	3	468.12	13.97	20.32	32.25	35.87	+ 0.2459	431
5	1	481.59	30.03	6.77	30.00	42.84	+ 0.7303	434
6	1	.....	....	...	....	....	.....	...
7	1	441.58	27.54	6.21	27.51	45.91	— 0.1834	397
8	1	.....	....	...	....	....	.....	...
9	2	461.21	10.24	72.52	11.00	31.69	+ 0.1344	429
10	2	461.21	10.24	72.52	11.00	34.20	+ 0.1539	426
11	2	461.21	10.24	72.52	11.00	30.43	+ 0.2018	430
12	2	461.21	10.24	72.52	11.00	30.96	+ 0.2163	429

nitrogen. This value, therefore, was used in correcting the urinary energy to nitrogen equilibrium.

Before pooling the 34 sets of data for statistical analysis, it was considered necessary to test for residual effects in passing from one diet period to another, possibly due to advancing age, small changes in environmental conditions or in management of the animals. An analysis of variance (Snedecor, '46) showed that residual effects were negligible.

The metabolizable energy values secured directly in the 34 tests comprising this experiment relate to the three experi-

mental diets, not to the three nutrients whose metabolizable energy values were desired. In order to get the desired information from the data secured, a multiple regression equation of the following type was fitted to the 34 sets of data by the method of least squares:

$$ME = cC + sS + fF$$ *

in which C, S and F are the grams of casein, starch and fat, on the dry basis, respectively, consumed per 10 days, ME is the corresponding metabolizable energy of the diet, and c, s, and f are the metabolizable energy per gram of casein, starch and fat, respectively. The evaluation of the latter constants by the method of least squares yielded the following equation:

$$ME = 4.673\ C + 3.962\ S + 8.770\ F.$$

Hence, the metabolizable energy values desired, with their standard errors, are: for casein 4.673 ± 0.089 cal. per gram; for starch 3.962 ± 0.016 cal. per gram; and for fat 8.770 ± 0.065 cal. per gram. The standard errors were computed by the method of Rider ('39, page 95).

The accuracy of these determinations is evident from the small size of their standard errors, and also from the multiple correlation coefficient, calculated to be + 0.992.

DISCUSSION

The multiple regression analysis of the determined metabolizable energy of the three experimental diets in order to estimate the metabolizable energy content of the three organic nutrients of which the diets were mainly composed is justified because (1) the three organic nutrients are independent components of the diets, (2) their proportionate occurrence differs widely in the three diets, and (3) there is no reason to suspect that the metabolizable energy content of any one of the nutrients is affected appreciably by the variable occurrence of the other two nutrients. The inclusion of two per cent of barium sulfate in the diets as roughage should introduce no appreci-

* A similar factorization procedure was used by Kraut et al. ('50) in determining the digestibility of animal and vegetable proteins from the digestibility by humans of the total proteins of 690 mixed diets.

able error in the factorization procedure adopted. The purpose of its inclusion was to promote regular laxation.

A factorization procedure of the metabolizable energy of the diets into their calorigenic nutrients seems justified by the fact that a large variation has been introduced for a single nutrient in the three rations, while keeping the rations completely balanced for maintenance. By including biologically inert barium sulfate instead of crude fiber which seems to yield energy to the rat (Shehata, '53) a doubtful calorigenic factor has been eliminated and at the same time roughage in the rations has been provided. It cannot be ruled out that the physiology of the gastro-intestinal tract, particularly the intestinal motility, may be affected by barium sulfate. However, such effects, if any, may be considered to be of little significance in the present experiment.

The metabolizable energy values which have been obtained for casein, starch and a mixture of fats for the rat are different from Atwater's calculated values for similar nutrients for man (table 10, Atwater, 1899). A fuel value of 4.67 cal. per gram of pure (moisture-free) casein is significantly greater than Atwater's reported mean value of 4.25 cal. per gram for the proteins of dairy products. This difference is in part due to a higher heat of combustion (5.83 cal./gm) of the casein used in the rat experiments than that of 5.65 cal./gm used by Atwater. Furthermore, Atwater found that for every gram of nitrogen in human urine resulting from a mixed diet there was unoxidized material sufficient to yield 7.9 cal. of energy. On the assumption that all the energy in the urine is derived from incompletely oxidized available protein, Atwater calculated that for every gram of protein digested by the human body 1.25 cal. ($7.9 \div 6.25 = 1.26$) of energy would be lost in the urine. In the case of the rat experimental data secured in this investigation have yielded a mean value of 6.29 cal. per gram of urinary nitrogen, when the rat metabolizes ingested protein calories from lean beef alone. Using this figure of 6.29 cal. and calculating protein calorie losses in urine on Atwater's as-

sumptions, it is found that 1.01 cal. of energy are lost in rodent nutrition for every gram of digested protein metabolized.

The metabolizable energy of the mixture of fats, 8.77 cal. per gram, is equivalent to a digestibility of 92% for the rat. Shehata ('53) computed a digestibility of 92% for lard for the rat. Atwater reported 95% "availability" (by which he means digestibility) of animal fats for humans.

The "fuel value" of 3.96 cal. per gram of starch corresponds to Atwater's 4.1 cal. per gram of cereal carbohydrates. As there are no losses of carbohydrate energy in normal metabolism, the "availability" coefficient of starch may be regarded as the apparent digestibility coefficient, i.e., 94%.

It may be pointed out in connection with the Atwater system of conversion factors that the first step in the derivation of the protein factors is the determination of the heats of combustion of pure proteins from the various classes of foods. Nevertheless, the factors are being applied not to the true proteins of foods but to conventional proteins obtained by multiplying the total nitrogen by 6.25 or some similar factor. Thus, the value of 5.65 applied to the true protein of meat will give a good estimate of the heat of combustion of this nutrient, but when applied to the conventional protein ($N \times 6.25$) of meat, it will give a falsely high value, since the non-protein nitrogenous compounds of meat do not have the same heat of combustion as meat protein; in fact, the main constituent of this group, creatine, is not oxidized in the animal body at all. Evidently for foods containing a considerable proportion of their nitrogen in non-protein forms, the metabolizable energy cannot conceivably be satisfactorily determined by the use of conversion factors such as those of Atwater. In such cases, direct determination must be made, or perhaps some variation of the multiple regression method used in this paper may be used.

SUMMARY

With the purpose of ascertaining the applicability of the Atwater energy conversion factors to rat nutrition, three balanced diets differing widely in their proportions of calori-

genic nutrients (casein, starch, and a mixture of fats) were fed to 12 adult rats according to a 3×3 latin square design, and the metabolizable energy for each diet for each rat was determined in 34 metabolism periods. The metabolizable energy values were then factorized into values for the three energy-yielding nutrients, using a multiple regression equation of the type: $ME = cC + sS + fF$, in which ME is the determined metabolizable energy for the diet, C, S and F the weights consumed of casein, starch and fat, respectively, and c, s and f the constants to be determined from the 34 sets of data by the method of least squares, representing the metabolizable energy per gram of the respective nutrients.

The metabolizable energy values thus obtained with their standard errors are as follows:

casein	4.673 ± 0.089 cal.
starch	3.962 ± 0.016 cal.
mixture of fats	8.770 ± 0.065 cal.

Analyses of the urine of rats on a very high-protein diet for energy and total nitrogen yielded 6.29 cal. per gram of urinary nitrogen. This value was used in correcting urinary energy to nitrogen equilibrium.

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INTERRELATION OF FAT, CARBOHYDRATE AND VITAMIN E IN THE DIET OF THE GROWING RAT [1]

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Gullickson and co-workers ('42) reported retarded growth and poor physical appearance of calves fed mineralized skim milk to which vegetable oils had been added in place of butterfat. In a more recent report by Gullickson et al. ('53) evidence was presented to indicate that the replacement of butterfat with vegetable oils in the diet of calves caused some symptoms characteristic of a vitamin E deficiency. These observations have stimulated renewed interest in the controversial issue regarding the comparative nutritive value of animal and vegetable fats. The literature pertaining to this subject is quite extensive and has been comprehensively reviewed elsewhere (Cowgill, '45; Deuel and Greenberg, '50). In brief, various groups of investigators who have used rats as experimental subjects have failed to reach concordant results with regard to the relative nutritive merits of butterfat and vegetable oils, and the reason for these discrepancies has never been satisfactorily explained.

The purpose of the present study was to assess the role which vitamin E might play in this problem particularly as related to type of fat and carbohydrate comprising the diet of the rat.

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EXPERIMENTAL

The basal ration was formulated to approach a composition intermediate between human and cows' milk and is a modification of the ration employed by Geyer et al. ('43). Each kilogram of ration contained casein [3] 180 gm, carbohydrate (lactose or sucrose) 480 gm, fat (corn oil [4] or butterfat [5]) 280 gm, salt mixture (Schultze, '50) 40 gm, and vitamin-casein mixture [6] 20 gm. Vitamin A and D were admiinstered orally in the form of haliver oil,[7] each rat receiving two drops per week. Supplements of vitamin E, where indicated, were provided by the daily oral administration of a drop of olive oil containing 0.3 mg α-tocopherol.[8] From the available evidence, Brown and Sturtevant ('49) have concluded that this level should satisfy the vitamin E requirements of the growing rat. All rations were prepared at intervals not exceeding one week and were stored at 4°C.

The rats used in this study were of a strain maintained in this laboratory for many years. Unless specified otherwise, young weanling rats weighing 35 to 45 gm were divided into groups of equal size with due regard for weight, sex and litter. Food and water were provided ad libitum,[9] and weights were recorded at weekly intervals.

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

[4] Mazola.

[5] Unsalted winter product obtained from the Department of Dairy Husbandry, University of Minnesota. The water was removed by melting the butter and separating the water from the oil phase in a separatory funnel.

[6] Composed of the following in milligrams: thiamine HCl 75, riboflavin 150, calcium pantothenate 250, inositol 500, *p*-aminobenzoic acid 1,000, 2-methyl-1,4-naphthoquinone 25, choline chloride 2,500, folic acid 10, biotin 5, pyridoxine 25, niacin 50, crystalline vitamin B_{12} 0.2 and casein to 100 gm. This vitamin mixture corresponds to the "high levels" of B vitamins which Boutwell et al. ('45) have shown to minimize the difference in nutritive value between corn oil and butterfat.

[7] Abbott Laboratories, North Chicago, Illinois. Each gram contains 60,000 U.S.P. units of vitamin A and 1,000 U.S.P. units of vitamin D.

[8] Merck and Co., Rahway, New Jersey.

[9] The authors are fully aware of the objections that have been raised to ad libitum feeding in studying this problem (Deuel et al., '44a) as well as the counter arguments advanced by others (Boutwell et al., '44; Nieman et al., '52). Although food consumption data are not presented in this paper, it was found that food consumption closely paralleled the rate of growth. It would be well to emphasize that the conclusions reached in this report are based on the premise that an increased requirement for food is the natural consequence of enhanced growth.

The degree of vitamin E deficiency induced by the various dietary modifications employed was ascertained by the hemolysis test described by György ('51). In this test the degree of hemolysis of red blood cells caused by dialuric acid is regarded as a measure of the severity of the vitamin deficiency. The hemolysis test was performed at the termination of each experiment on blood taken from the tail.

TABLE 1

Growth response of young from mothers of unknown dietary history

DIETARY COMPONENT		WEIGHT GAIN [1]	
Fat	Carbohydrate	− E	+ E
		gm	*gm*
Corn oil	Lactose	88.7 ± 4.2	110.5 ± 5.6
Butterfat	Lactose	108.5 ± 3.6	108.7 ± 4.0
Corn oil	Sucrose	169.3 ± 7.6	165.2 ± 3.9
Butterfat	Sucrose	166.3 ± 7.4	169.8 ± 6.8

[1] Mean of each group (6 males) ± the standard error of the mean over a period of 6 weeks.

In certain instances the level of α-tocopherol in the blood serum was determined by the method of Lowry and Bessey ('46). For this purpose, animals were exsanguinated by decapitation, the blood allowed to clot in the collecting vessel, and the serum separated by centrifugation.

Statistical analyses of the data were made according to the methods described by Snedecor ('46).

RESULTS

Growth of young from mothers of unknown dietary history. The initial experiments were conducted with young weanling males taken from mothers used in other unrelated experiments currently in progress. The importance of the maternal diet was not realized at the time, so no record was kept relating each litter to its respective mother. In table 1 are shown the data pertaining to the growth of these animals when placed on rations containing corn oil or butterfat with lactose or

sucrose as sources of carbohydrate in the presence or absence of supplemental vitamin E. The failure of corn oil to support the same rate of growth as butterfat is apparent when lactose constitutes the source of carbohydrate in the absence of supplemental vitamin E ($t = 3.54$; $P < 0.01$).[10] When a vitamin E supplement was fed the difference between these two fats was no longer evident. This follows from the observation that vitamin E added to the corn oil-lactose ration stimulated growth ($t = 3.03$; $P < 0.02$) but was ineffective when added to the butterfat-lactose ration. Growth was markedly improved in all instances when lactose was replaced by sucrose, but under these conditions no differences between corn oil and butterfat were observed either in the presence or absence of vitamin E.[11]

An experiment was also conducted in which corn oil and a hydrogenated vegetable oil,[12] in the presence of lactose or sucrose, were compared with respect to the level of α-tocopherol in the blood serum. Supplemental vitamin E was not provided in this particular study. A number of animals were sacrificed from each group at weekly intervals over a period of 5 weeks, and the blood sera were analyzed for α-tocopherol. The data in table 2 show that the level of α-tocopherol in the blood serum of animals receiving the corn oil-lactose ration had dropped to almost one-quarter of its initial value within three weeks. On the other hand, the serum tocopherol values of rats consuming corn oil with sucrose, or the hydrogenated vegetable oil with either lactose or sucrose, remained fairly constant over the 5-week experimental period with no significant differences between these three rations. Because of the

[10] Although detailed growth data covering the entire experimental period have not been included in table 1, it should be pointed out that the inferiority of the corn oil diet became apparent after the first week and remained so throughout the period of observation. Diarrhea was occasionally observed in the lactose-fed rats but gradually diminished and had disappeared by the second week of the experiment.

[11] In experiments not reported here essentially the same growth pattern was obtained on diets containing 28% casein, equivalent to the protein content of whole milk powder.

[12] Crisco.

decrease in the number of animals as the experiment progressed, no reliable growth data were available from this particular study. The marked decrease in serum tocopherol observed in the case of the corn oil-lactose ration is consistent, however, with the growth data presented in table 1, in which inferior growth on the same ration is recorded.

Growth of young from mothers receiving a vitamin E deficient ration. Subsequent attempts to reproduce the results of these initial experiments frequently failed to disclose any

TABLE 2

Blood serum α-tocopherol[1] *as related to the type of fat and carbohydrate in the diet*

TIME	CORN OIL		HYDROGENATED VEGETABLE OIL	
	+ lactose	+ sucrose	+ lactose	+ sucrose
weeks				
1	225 ± 7.1 (5)	290 ± 18.3 (5)	280 ± 13.1 (5)	310 ± 12.3 (5)
2	150 ± 20.4 (3)	275 (1)	217 ± 7.5 (2)	265 (1)
3	75 ± 10.8 (3)	310 ± 30.9 (4)	255 ± 13.7 (4)	240 ± 22.2 (4)
4	85 ± 7.1 (3)	350 ± 10.8 (3)	250 ± 4.1 (3)	260 (1)
5	87 ± 9.2 (7)	334 ± 5.8 (5)	370 ± 23.5 (5)	310 ± 18.2 (5)

[1] Expressed as μg/100 ml ± standard error of the mean. The number of animals used for calculating each mean value is indicated in parentheses. The initial mean level of α-tocopherol before animals were separated into various groups was 300 ± 12.7 based on 8 animals.

differences in growth between rats receiving corn oil and butterfat such as had been observed previously when lactose was employed in the absence of supplemental vitamin E. The most likely explanation was the probability that some of the animals used in these later studies were not E-deficient at the outset of the experiments and hence were refractory to further vitamin E supplementation. To achieve a consistency of response, experiments were conducted using only young animals which had been depleted of vitamin E.

Litters raised by mothers receiving the vitamin E-deficient ration described by György ('51) since parturition were divided at weaning (21 days) into two groups of equal sex distribution. Only those animals showing definite signs of vitamin

E deficiency were so selected. These symptoms included a sub-normal weaning weight (20 to 30 gm), muscular paralysis affecting the hind-legs, and hemolysis of the red blood cells by dialuric acid. Littermate comparisons were made between corn oil and butterfat as modified by lactose or sucrose in the presence and absence of supplemental vitamin E. Hemolysis tests were again conducted at the end of 6 weeks when the experiment was terminated.

The results of this experiment, presented in table 3, confirm the superiority of butterfat over corn oil in diets containing lactose from which supplemental vitamin E had been withheld. No differences between corn oil and butterfat were noted when additional vitamin E was supplied to rats receiving rations containing either lactose or sucrose.

The hemolysis data showed that the unsupplemented rations were in all cases inadequate for protection of the red blood cells against hemolysis by dialuric acid. The hemolysis observed with the blood of rats in groups 1b, 3a and 3b is somewhat unexpected in view of the fact that these rations appeared to provide sufficient vitamin E to meet the requirements for growth. A possible explanation is that the rat's requirement of vitamin E for growth may be less than that needed for protection against hemolysis by dialuric acid. This conclusion is supported by the observation of Rose and György ('50) who found that a daily dose of 0.5 mg α-tocopherol was inadequate for complete protection against hemolysis. The protective dose against hemolysis must therefore be at least 50% greater than that required for growth if the latter requirement is taken to be 0.3 mg/rat/day (Brown and Sturtevant, '49).

The fact that the hemolysis values on the unsupplemented corn oil rations were generally lower than those on the rations containing butterfat is not readily explainable. It may be that other forms of tocopherol, or the products resulting from the oxidative destruction of tocopherol (see discussion), are relatively more effective in preventing hemolysis than meeting the requirements for growth. Until more information is available regarding the specificity of the hemolysis test, it would be

TABLE 3

Growth response and hemolysis data of young from mothers receiving a vitamin E-deficient ration

SERIES	GROUP [1]	DIETARY COMPONENT		VITAMIN E SUPPLEMENT	NO. OF ANIMALS	WEIGHT GAIN [2] IN 6 WEEKS	"t" VALUE [3]	HEMOLYSIS [2]
		Fat	Carbohydrate					
				mg/day		*gm*		%
1	a	Corn oil	Lactose	0	13	75.0 ± 5.7	4.8*	21 ± 8
	b	Butterfat	Lactose	0	13	91.7 ± 3.8		70 ± 6
2	a	Corn oil	Lactose	0.3	14	88.4 ± 3.6	0.5	0
	b	Butterfat	Lactose	0.3	14	85.9 ± 2.1		0
3	a	Corn oil	Sucrose	0	13	113.4 ± 2.2	1.2	9 ± 3
	b	Butterfat	Sucrose	0	13	117.8 ± 4.1		57 ± 6
4	a	Corn oil	Sucrose	0.3	9	111.1 ± 4.3	1.9	0
	b	Butterfat	Sucrose	0.3	9	119.0 ± 5.7		0

[1] Groups a and b within each series involve a comparison between littermates.

[2] Mean of each group ± the standard of the mean.

[3] Calculated on the basis of paired comparisons between littermates of the same initial weight (± 1 gm) and sex. "t" value with asterisk is significant at $P < .01$.

unwise to attach too much significance to these data as a measure of the vitamin E status of the rat.

Growth of rats depleted of vitamin E with tri-o-cresyl-phosphate (TOCP). Further evidence on the relation of vitamin E to the nutritive value of corn oil and butterfat was sought by using animals depleted of vitamin E through the ingestion of a suitable antagonist. Draper et al. ('52) have shown that TOCP increases the dietary requirement for vitamin E in the rat when incorporated into a synthetic ration containing 2% sulfathalidine.[13] Young weanling rats from

TABLE 4

Growth response and hemolysis data of young rats depleted of vitamin E by tri-o-cresyl phosphate and sulfathalidine

DIETARY COMPONENT		WEIGHT GAIN [1] IN 6 WEEKS		HEMOLYSIS	
Fat	Carbohydrate	−E	+E	−E	+E
		gm	*gm*	%	%
Corn oil	Lactose	43.0 ± 5.0	52.3 ± 2.7	64 ± 10	0
Butterfat	Lactose	71.8 ± 6.9	74.1 ± 8.2	72 ± 11	0
Corn oil	Sucrose	102.1 ± 12.5	102.5 ± 2.6	2 ± 1	0
Butterfat	Sucrose	117.7 ± 7.2	116.3 ± 8.2	48 ± 8	0

[1] Mean of each group (8 males) ± the standard error of the mean.

mothers on a stock ration were placed on the ration described by Draper et al. ('52) containing 2 mg TOCP [14] per gram for a period of two weeks. Depressed growth, rough haircoat, diarrhea, and positive hemolysis characterized the symptoms of these animals at the end of this preliminary depletion period. A total of 64 depleted animals were randomized into 8 groups duplicating the design of the experiment shown in table 1. The results of this experiment are summarized in table 4.

The nutritive superiority of butterfat over corn oil in the absence of supplemental vitamin E is highly significant with the lactose ration ($t = 3.41$; $P < 0.01$) and somewhat less significant with the sucrose ration ($t = 2.23$; $P < 0.05$). When

[13] Trade name for phthalylsulfathiazole produced by Sharpe and Dohme, Inc., Philadelphia, Pa.

[14] Eastman Organic Chemicals, Distillation Products Inc., Rochester, N. Y.

the effects of vitamin E supplementation are considered, it may be seen that only where the corn oil-lactose ration was supplemented with vitamin E was there a partial improvement in growth performance ($t = 2.03$; $P < 0.05$). It is important to note that, in contrast to the results of previous experiments, butterfat now retains its superiority over corn oil even on the vitamin E supplemented rations containing either lactose ($t = 2.56$; $P < 0.05$) or sucrose ($t = 2.42$; $P < 0.05$). One is led to conclude, therefore, that, under the conditions wherein vitamin E is no longer limiting, some other factor must account for the superior growth performance of rats receiving butterfat.

The hemolysis data obtained in this experiment confirm the previous observation that hemolysis may be demonstrated in spite of the fact that sufficient amounts of vitamin E appeared to be available for growth on the unsupplemented rations containing butterfat and lactose, and corn oil or butterfat and sucrose. In agreement with previous data, the replacement of corn oil in the unsupplemented rations with butterfat again produced higher hemolysis values.

The effect of sulfathalidine

In attempting to account for the results of the TOCP experiment which indicated that butterfat was superior to corn oil even when adequate amounts of vitamin E were available for growth, the possibility that the sulfathalidine, which had been included in the depletion ration, might be responsible for this effect was considered. Accordingly, basal rations containing corn oil or butterfat and lactose or sucrose were modified by adding 2% sulfathalidine at the expense of the carbohydrate component; α-tocopherol was incorporated into all the diets at a level of 10 mg per 100 gm ration. The adequacy of this level of vitamin E was demonstrated by the negative hemolysis test which was obtained in all instances at the completion of the experiment. The data pertaining to the growth of young weanling rats (from mothers on a stock ration) placed on these various rations are shown in table 5. If these data are com-

pared to the growth which was obtained on similar rations without sulfathalidine (table 1, column "+ E"), it is apparent that sulfathalidine has depressed growth to a considerable extent. The data of table 5 show moreover that the replacement of corn oil by butterfat has resulted in a partial counteraction of this growth depression to a significant extent on both the lactose and sucrose rations.

TABLE 5

Effect of sulfathalidine on growth of rats receiving corn oil or butterfat and lactose or sucrose

DIETARY COMPONENT		NO. OF ANIMALS	WEIGHT GAIN [1]	"t" VALUE [2]
Fat	Carbohydrate			
			gm	
Corn oil	Lactose	11	52.9 ± 1.6	3.1
Butterfat	Lactose	11	65.5 ± 3.6	
Corn oil	Sucrose	12	97.4 ± 3.4	5.1
Butterfat	Sucrose	12	120.7 ± 4.9	

[1] Mean ± the standard error of the mean over an experimental period of 6 weeks.

[2] Calculated on the basis of paired comparisons between littermates of the same initial weight (± 1 gm) and sex. Both "t" values indicate a level of significance of $P < 0.01$.

DISCUSSION

From the data presented here it appears that corn oil does not support the same rate of growth as butterfat under conditions where (1) supplemental vitamin E is withheld from a ration containing lactose as the source of carbohydrate, and (2) sulfathalidine is incorporated into a basal ration containing either lactose or sucrose and all the known dietary essentials.

It is a curious fact that vegetable oils have been generally considered to be rich sources of vitamin E as compared to animal fats.[15] Not to be overlooked, however, is the antago-

[15] According to Lange ('50) the α-tocopherol contents of butterfat and corn oil are 3 and 9 mg/100 gm respectively. In addition, corn oil contains 81 mg/100 gm γ-tocopherol. Independent analyses by one of us (J. E. G.) on the fats used in the present study gave comparable values.

nistic effect which the highly unsaturated fatty acids, present to a considerably greater extent in vegetable oils, are known to exert on the availability or utilization of vitamin E (Mason and Filer, '47). This effect was well illustrated by the data of table 2 which showed that the replacement of a hydrogenated vegetable oil by corn oil in a lactose-containing ration led to a marked decrease in the α-tocopherol level of the blood serum. Also significant in this respect is the report by Hirsch and Jacquot ('49) that sunflower seed oil at a level of 15% produced symptoms of a vitamin E deficiency in the rat in spite of the high level of tocopherol known to be present in this oil.

Also to be considered is the fact that corn oil accentuates the need for vitamin E only when lactose constitutes the source of carbohydrate in the diet. Pertinent, perhaps, is the observation by Bergheim et al. ('45) that, of a number of dietary components tested for their effect on the redox potential of the intestinal tract, only lactose led to a marked increase. It may be that the elevated redox potential produced by lactose in combination with the unsaturated fatty acids originating from corn oil act synergistically to accelerate the oxidative destruction of tocopherol in the intestinal tract.

It is unlikely that vitamin E can be implicated in the failure of various investigators to reach agreement concerning the comparative nutritive merits of butterfat and vegetable oils. A critical examination of the expeimental rations used by the various workers studying this problem has shown that adequate supplementation with vitamin E had been employed in all instances.[16]

Apparently unrelated to vitamin E is the superiority of butterfat over corn oil for the support of growth when sulfathalidine was incorporated into the basal ration, an effect which is observed when either lactose or sucrose provide the

[16] Experiments were conducted by the present authors (unpublished) to duplicate as closely as possible the experimental conditions employed by the Wisconsin (Boutwell et al., '43) and California (Deuel et al., '44b) groups in which corn oil or butterfat were added to mineralized skim milk. No difference between these two fats was observed, a finding which agrees with that of the California group.

carbohydrate portion of the ration. One possible interpretation of the data at hand is that sulfathalidine has modified the intestinal flora in such a way that some unidentified nutrient normally synthesized by certain intestinal bacteria is no longer available to the host. Corn oil may be unable to supply this missing nutrient, whereas butterfat, or some component intimately associated with it, may be able to provide it to a limited extent. Others have suggested the existence of a growth promoting factor in butter, although attempts to isolate and identify it have not met with any degree of success (Nath et al., '48).

The modifying influence of a bacteriostatic substance such as sulfathalidine on the response of rats to various fats serves to emphasize once again the important role which the intestinal flora plays in nutritional studies of this nature (Elvehjem, '48). Failure to recognize or control differences in the microbial population of various strains of rats could conceivably lead to the type of disparity in results which has thus far characterized this field of investigation.

SUMMARY

The growth-promoting properties of corn oil and butterfat for the vitamin E-depleted rat have been compared using lactose or sucrose as sources of carbohydrate both in the presence and absence of supplemental vitamin E. In the absence of supplemental vitamin E and with lactose constituting the carbohydrate component of the diet, butterfat supported a rate of growth which exceeded that produced by corn oil. With the administration of vitamin E, however, no demonstrable difference between the two fats was evident on either the lactose- or sucrose-containing rations.

Young rats depleted of vitamin E by preliminary feeding for two weeks on a ration containing tri-o-cresyl phosphate and sulfathalidine likewise grew better when butterfat replaced corn oil on lactose diets which were not supplemented with vitamin E. Some improvement in growth performance on the corn oil-lactose diet was effected by supplementation with

vitamin E. The nutritive superiority of butterfat over corn oil, was retained, however, even in the presence of supplemental vitamin E on rations containing either lactose or sucrose. The incorporation of sulfathalidine into rations complete with respect to vitamin E resulted in depressed growth which could be partially overcome by replacing corn oil with butterfat. Whether or not these observations are due to known components of butterfat cannot be decided on the basis of present evidence.

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A MECHANISM OF THE VITAMIN-SPARING EFFECT OF ANTIBIOTICS[1]

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It has been clearly established that when animals are grown on diets containing suboptimum amounts of certain vitamins, improved growth can be obtained by supplementing the diets with antibiotics. This response has been shown in the rat by Linkswiler et al. ('51), Lih and Baumann ('51), Guzman-Garcia, Sarles and Baumann ('53) and Sauberlich ('52) and in the chick by Biely and March ('51), Coates et al. ('51), Waibel et al. ('52) and Oleson et al. ('50). It is the purpose of the present report to provide more information on the mechanism of action of the vitamin-sparing effects of antibiotics.

EXPERIMENTAL

Straight-run (New Hampshire ♂♂ × single-comb White Leghorns ♀♀) cross-bred chicks, which were the progeny of hens fed diet B_1 described previously (Robblee and co-workers, '48), were used in all studies. The chicks were housed in elec-

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trically heated, brooder-type batteries with raised screen floors. Feed and water were supplied ad libitum. The chicks were wing-banded and weighed at one day of age. Weights were recorded at weekly intervals.

The chicks were divided according to weight, into groups of 30 each in experiment 1, and 15 each in experiment 2, and immediately placed on test for 4 weeks. The semi-purified basal ration contained carbohydrate 60.2 gm, alcohol-extracted casein 18 gm, gelatin 10 gm, salts V (Briggs et al., '43) 6 gm, soybean oil 5 gm, L-cystine 0.5 gm, thiamine hydrochloride 0.6 mg, riboflavin 1.2 mg, nicotinic acid 10 mg, pyridoxine hydrochloride 0.8 mg, calcium pantothenate 4.0 mg, choline chloride 170 mg, biotin 0.06 mg, inositol 100 mg, vitamin B_{12} 0.005 mg, para-aminobenzoic acid 10 mg, 2-methyl-1,4-naphthoquinone 0.05 mg, and α-tocopherol acetate 1 mg. Fortified haliver oil (60,000 U.S.P. units of vitamin A, 6,000 U.S.P. units of vitamin D_3 per gram) was given by dropper (two drops per chick per week). All chicks were also supplemented with two drops of freshly prepared thiamine hydrochloride (10 mg/ml) at the end of the first and second weeks.

The high folic acid diets contained 500 µg folic acid/100 gm diet and the low folic acid diets contained 25 µg folic acid/100 gm diet when sucrose was the carbohydrate and no folic acid when dextrin was the carbohydrate. Since Luckey et al. ('46) had shown a decreased folic acid requirement on dextrin diets, it was thought best to run these groups with no dietary folic acid in order to measure the growth response with antibiotics.

In experiment 1 chicks were taken at various times throughout the experiment for bacteriological studies, but in experiment 2 chicks were taken only at the termination of the experiment.

Intestinal bacteria were counted and coliforms isolated, grown in the synthetic medium of Waring and Werkman ('43), modified by the addition of ferric ammonium citrate, and the cells removed by centrifugation. The folic acid content of the supernatant was determined by the method described by Dietrich et al. ('49), using *Streptococcus faecalis* as the test

organism. A detailed study of the bacteriological work will be reported separately. In the first experiment the contents of the various parts of the intestinal tracts of three chicks from each group were pooled. Coliforms were isolated from the ileum, duodenum and cecum contents.

The amount of folic acid in the supernatant liquid of 48-hour cultures was used for the comparisons reported. The 48-hour incubation period was selected from a trial in which replicate samples of representative coliform isolates were incubated for various time intervals up to 72 hours. In experiment 2 the coliforms from the ileum contents of each of three birds were isolated and the individuality of the chicks maintained. The livers of 5 other chicks were removed and assayed for folic acid by the procedure reported previously (Dietrich et al., '49). The intestines of the same 5 chicks were removed from the base of the gizzard to the ileocecal junction, excluding the cecum, and the intestinal contents were flushed out, collected, homogenized in a Waring Blendor, made up to volume and autoclaved for 10 minutes at 15 lb. of pressure. An aliquot was taken for dry-weight determination and the rest was filtered and assayed for folic acid as indicated above.

RESULTS AND DISCUSSION

It is apparent from the growth data in table 1 that the groups supplemented with antibiotics, whether on the sucrose- or the dextrin-containing diets, grew better than those not supplemented with the antibiotics. Both the aureomycin and the bacitracin-penicillin supplements were active but the latter always produced slightly heavier chicks. Folic acid was the most limiting nutrient in these diets; therefore it is reasonable to assume that the growth improvement was due in part to the sparing of folic acid by the antibiotic. The lack of a complete response on the dextrin diets to antibiotic supplements was probably due to the absence of folic acid from the diet. A trace of the vitamin may be necessary to obtain improved growth with antibiotics.

Differences in the amounts of folic acid produced by isolated coliforms, *in vitro*, were apparent after 11 days, as is shown in table 2. The differences were greatest among organisms isolated from the ileum contents. After 29 days, approximately three times as much folic acid was produced by organisms isolated from the ileum contents of supplemented chicks than was produced by those from the ileum contents of control chicks. There appeared to be no difference in the amount of folic acid produced by isolates obtained from the cecum contents of the chicks. The amount of folic acid produced by coliforms from

TABLE 1

Effect of antibiotics on the growth of chicks fed low folic acid diets

DIETS FED	WEIGHT AT 4 WEEKS
	gm
Sucrose + 25 μg folic acid	284 (21)[1]
Sucrose + 25 μg folic acid + 5 mg % aureomycin	338 (24)
Sucrose + 25 μg folic acid + 2.5 mg % bacitracin + 10 mg % penicillin	354 (27)
Dextrin (folic acid deficient)	210 (21)
Dextrin + 5 mg % aureomycin	250 (27)
Dextrin + 2.5 mg % bacitracin + 10 mg % penicillin	258 (29)

[1] Figures in parentheses represent the number of birds used.

the ceca of the control chicks at 11 days was low but the variation was too large to give the value much significance. The level of folic acid produced by cecal isolates from all groups was approximately the same as that produced by the ileal isolates from antibiotic-supplemented groups. The coliforms from the cecum contents of the control chicks apparently had a greater capacity to synthesize folic acid than did the coliforms from the ileum contents of these chicks.

The coliforms isolated from the dextrin controls produced significantly more folic acid than did those from the sucrose controls. This could explain the decreased requirement for dietary folic acid of chicks receiving dextrin diets (Luckey et al., '46). There is a trend toward still higher folic acid production by coliforms isolated from chicks receiving bacitracin-

TABLE 2

Effect of antibiotics on the in vitro production of folic acid by intestinal coliforms[1]

DIETS FED	DAYS ON EXP.	CONTROL		+ AUREOMYCIN		+ BACIT. + PEN.	
		Ileum	Cecum	Ileum	Cecum	Ileum	Cecum
Sucrose + 25 μg folic acid/100 gm diet	11	1.72 ± 0.64 (6)	2.92 ± 1.49 (6)	5.32 ± 0.20 (5)	4.84 ± 0.23 (7)	...	...
	23	...	5.36 ± 0.66 (10)	5.63 ± 0.14 (5)	5.04 ± 0.28 (5)	...	5.06 ± 0.43 (6)
	29	1.78 ± 0.14 (11)	5.07 ± 0.51 (6)	5.28 ± 0.35 (6)	6.24 ± 0.47 (5)	5.07 ± 0.81 (5)	7.63 ± 1.55 (6)
Dextrin, folic acid deficient	29	5.25 ± 0.59 (10)	...	6.46 ± 0.17 (7)	...	7.66 ± 0.44 (12)	...

[1] Values given are millimicrograms per milliliter of supernatant, including standard error $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.
Values in parentheses indicate number of isolates. Level of antibiotics as in table 1.

penicillin supplements, although the difference is not as great as in the sucrose-fed groups.

To determine how rapidly this difference in folic acid production was established, 6 4-week-old control chicks from the experiment reported above were fed bacitracin-penicillin-supplemented diets for 5 days and one bird was sacrificed each day (zero to 5 days). Table 3 shows that by the second day coliforms were isolated that produced an increased amount of folic acid. The level of folic acid produced by the coliforms isolated from the duodenum contents increased by the third

TABLE 3

Length of time required to change to a high folic acid-producing intestinal flora

DAYS ON ANTIBIOTICS	ILEUM	DUODENUM	CECUM
	mμg/ml	*mμg/ml*	*mμg/ml*
0	2.98 ± 0.1 (9)[1]	3.24 ± 0.45 (7)	5.67 ± 0.56 (6)
1	3.18 ± 0.46 (12)	4.58 ± 0.43 (13)	...
2	5.05 ± 0.77 (6)	3.64 ± 0.64 (5)	7.01 ± 1.25 (6)
3	5.04 ± 0.25 (10)	5.41 ± 0.20 (7)	...
4	4.77 ± 1.07 (6)	...	6.29 ± 0.86 (9)
5	9.76 ± 1.21 (6)	6.24 ± 0.85 (4)	6.06 ± 0.68 (9)

[1] Figures in parentheses indicate the number of isolates. Standard error included $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

Bacitracin-penicillin-supplemented as in table 1.

day and the level of folic acid produced by isolates from the cecum contents remained constant.

Table 4 shows the results of experiment 2, which was designed to study the above phenomena in more detail. The growth data are similar to those obtained in experiment 1. The bacitracin-penicillin-supplemented group grew as well as did the high folic acid group. It is apparent that the increased production of folic acid by coliforms isolated from the ileum contents of chicks supplemented with antibiotics can be correlated with increased liver storage of the vitamin but not with increased intestinal folic acid. The liver folic acid and the amount of folic acid produced by the coliforms *in vitro* were both approximately doubled when antibiotics were fed. Gug-

TABLE 4

Effect of antibiotics on liver, intestinal and in vitro production of folic acid [1]

DIETS FED	LIVER FOLIC ACID	INT. FOLIC ACID	FOLIC ACID PRODUCED BY ISOLATES	% OF ISOLATES GREW	WEIGHT AT 4 WEEKS
	μg/gm	*μg/gm dry wt.*	*mμg/ml*		*gm*
Sucrose basal + 500 μg folic acid			4.90 ± 0.71 (7)	21	354 (15)
Sucrose basal + 25 μg folic acid	0.74 ± 0.14 (5)	0.52 ± 0.02 (5)	4.20 ± 0.14 (5)	30	289 (15)
Sucrose basal + 25 μg folic acid + aureomycin	1.44 ± 0.14 (5)	0.51 ± 0.05 (5)	8.76 ± 1.08 (18)	95	339 (15)
			...	0	
			4.20 ± 0.10 (12)	100	
Sucrose basal + 25 μg folic acid + bacit. + pen.	1.62 ± 0.30 (5)	0.51 ± 0.04 (5)	5.19 ± 0.22 (12)	100	350 (15)
			7.46 ± 0.17 (17)	100	
			7.73 ± 0.50 (10)	59	

[1] Figures in parentheses indicate number of isolates. Standard error included $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$. Level of antibiotics as in table 1.

genheim et al. ('53) recently reported that rats fed diets low in riboflavin or pantothenic acid had increased liver and urinary levels of the vitamins when their diets were supplemented with antibiotics. The lack of an increase in intestinal folic acid is probably due to a steady state that is maintained in the intestine due to rapid absorption of the vitamin as it is made available by the microorganisms. All values were lower than those reported by Monson et al. ('52), due undoubtedly to the lower dietary intake of folic acid.

The individuality of the chicks was maintained in this experiment. A large percentage of coliforms from chicks receiving no antibiotics did not grow in the synthetic medium. These data are found in table 4. Only 21% of the coliforms from the high folic acid and 30% of the coliforms from the low folic acid controls grew in the synthetic medium. Failure of the coliform isolates to grow in the synthetic medium had not been encountered in experiment 1. The results obtained from isolates from all three chicks from each of these two groups were combined so that standard errors could be calculated. Most of the coliforms from two of the aureomycin-supplemented chicks grew, whereas none of the isolates from a third chick grew. It is interesting that this chick weighed the least of the three (288 gm, as compared to 493 gm and 412 gm). The majority of the isolates from the intestinal contents of the bacitracin-penicillin-supplemented group grew. It would appear that there is some relationship between antibiotic supplementation, or its effectiveness, and the ability of the isolated coliforms to grow in this synthetic medium.

Although the amount of folic acid produced by the isolates from the intestinal contents of control groups was greater in this experiment than in experiment 1, the coliform from one of the chicks from the aureomycin-supplemented group, and all of the chicks from the bacitracin-penicillin-supplemented group, produced more folic acid than did those from the controls.

Many workers have reported that antibiotics cause changes in the intestinal flora of chicks. This work has been reviewed

by Branion et al. ('53). Anderson et al. ('52) found, in addition to other results, that penicillin supplementation caused the appearance of an atypical strain of *Escherichia coli* in the intestines of chickens. Guzman-Garcia, Sarles and Baumann ('53) showed that penicillin added to a synthetic ration caused increases in the numbers of coliform bacteria in the contents of both upper and lower parts of the intestinal tract of the rat.

The work reported here indicates that the increased growth obtained by supplementing diets low in folic acid with antibiotics may be due, at least in part, to the appearance of intestinal coliforms that produce increased amounts of folic acid. These organisms become established in the intestinal tract very rapidly. The organisms are beneficial to the host by producing more folic acid which is available for growth as evidenced by liver levels. It is significant that the largest differences in the amount of folic acid synthesized were obtained among isolates from an area of the digestive tract in which active absorption occurs.

SUMMARY

The growth of chicks fed synthetic diets containing limiting amounts of folic acid was increased by supplementing the diets with antibiotics. This increase in growth was accompanied by the appearance of one or more coliforms in the ileum and duodenum contents that produced increased amounts of extracellular folic acid. This change was apparent in two days in the ileum contents of 4-week-old chicks. It was also observed that the increased folic acid production was correlated with increased liver folic acid, but there was no change in the concentration of intestinal folic acid. These results explain at least in part the mechanism by which antibiotics spare vitamins for the chick.

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THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

XI. THE PROTECTIVE EFFECT OF VARYING LEVELS OF ETHYL LINOLEATE AGAINST MULTIPLE SUBLETHAL DOSES OF X-IRRADIATION IN THE RAT [1, 2, 3]

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ONE FIGURE

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The protection afforded by fat to rats given a series of sublethal doses of x-irradiation was reported earlier ('52) by Cheng and co-workers. In these original observations, it was found that the protection afforded by diets containing 2% of cottonseed oil was as satisfactory as that given by regimens in which 15 or even 30% of this foodstuff was present. It was likewise noted that, in the case of relatively old rats, both sexes were protected equally well by fat, whereas in the case of young sexually mature rats, best protection could be demonstrated in the males.

It was later reported from this laboratory (Deuel et al., '53) that a daily supplement of methyl linoleate as small as 10 mg was able to extend the survival time of male rats exposed to x-irradiation over and above that of animals on

[1] This work was carried out under a contract between the University of Southern California and the Atomic Energy Commission (No. AT(11-1)-113).

[2] Contribution 355 of the Department of Biochemistry and Nutrition, University of Southern California.

[3] The results were presented in part at the Gordon Research Conference, August 10, 1953.

the same fat-low regimen, but which were not supplemented with linoleate.

In view of the satisfactory protective action against x-irradiation afforded by diets containing only 2% of cottonseed oil, and especially of supplements of only 10 mg of methyl linoleate, one might well inquire whether or not the

TABLE 1

Composition of low-fat diet used for the production of essential fatty acid deficiency in rats

DIETARY CONSTITUENTS	AMOUNT
	%
Casein, vitamin test (General Biochemicals, Inc., Chagrin Falls, Ohio)	20.0
Sucrose (Commercial)	70.68
Salt mixture [1] (General Biochemicals Co., Inc., Chagrin Falls, Ohio)	4.00
Cellulose (Solka-floc from The Brown Co., San Francisco, Calif.)	4.00
Fat-soluble vitamin mixture [2]	1.00
Water-soluble vitamin mixture [3]	0.32

[1] L. G. Wesson, Science, *75:* 339–340 (1932).

[2] The fat-soluble vitamin mixture was composed of the following vitamins made up to 100 ml with propylene glycol: Napsol, 8.72 gm (vitamin A: 100,000 U.S.P. units per gram and vitamin D_2: 20,000 I. U. per gram) (National Oil Products Co., Harrison, New Jersey) and mixed tocopherol 25.7 gm (Distillation Products, Inc., Rochester, New York).

[3] The water-soluble vitamin mixture had the following composition: choline chloride, 67.51%; thiamine hydrochloride, 2.43%; riboflavin, 0.92%; pyridoxine hydrochloride, 0.91%; calcium pantothenate, 2.02%; niacin, 2.02%; *i*-inositol, 16.85%; folic acid, 0.34%; biotin, 0.08%; vitamin B_{12}, 0.01%; ascorbic acid, 6.74%; and menadione, 0.17%.

beneficial effect of fat depends upon the presence of only catalytic amounts of linoleate. It has been found that, although growth is stimulated in male rats on a fat-low diet by amounts of methyl linoleate as small as 5 mg (Greenberg et al., '50), the optimum level of this essential fatty acid probably exceeds 200 mg per day (Deuel et al., '51). The present studies were designed to determine whether or not the protective action of essential fatty acids is related to

the amount ingested in the same manner as growth is related to this factor, or whether only catalytic amounts are required to initiate this action.

EXPERIMENTAL

The tests were carried out on young male albino rats of the University of Southern California strain. The animals were kept in separate wire-bottom cages and were fed the fat-low diet ad libitum from weaning until they had been depleted as judged by a plateau in weight, and by the appearance of essential fatty acid deficiency symptoms. This diet has been repeatedly employed successfully in this laboratory for producing such deficiency. The composition of the diet is given in table 1.

The animals were weighed weekly. At depletion, after approximately 9 to 12 weeks, the rats were distributed evenly among 4 groups. These groups were maintained on the same fat-low diet and, in addition, received the following supplements daily: group I (negative control), ethyl linoleate,[4] 0 mg, ethyl laurate,[5] 0.1 ml; group II, ethyl linoleate, 10 mg, ethyl laurate, 0.1 ml; group III, ethyl linoleate, 50 mg, ethyl laurate, to 0.15 ml; and group IV, ethyl linoleate, 100 mg, ethyl laurate, to 0.3 ml. Ethyl laurate was used as a diluent to facilitate the measurement of accurate quantities of the ethyl linoleate. Supplements were administered orally twice weekly for 8 weeks, by means of a syringe with a blunt needle. After this preliminary 8-week supplementation period, x-irradiation was begun. Each group was subjected to doses of 200 *r* per week for 10 weeks. Supplementation was continued during and after the period of x-irradiation, until the termination of the experiment. The radiation was carried out in a manner similar to that employed in our earlier tests (Cheng et al., '52).

[4] Prepared by Dr. Willy Lange of Procter and Gamble Co., Cincinnati, Ohio.

[5] Eastman Kodak Co., Rochester, N. Y.

RESULTS

The percentage survival of the rats receiving no ethyl linoleate or receiving the essential fatty acid ester at several levels for a 13-week period is plotted in figure 1. It will be noted that the percentage survival is greater in all cases for the rats receiving ethyl linoleate than for those which received

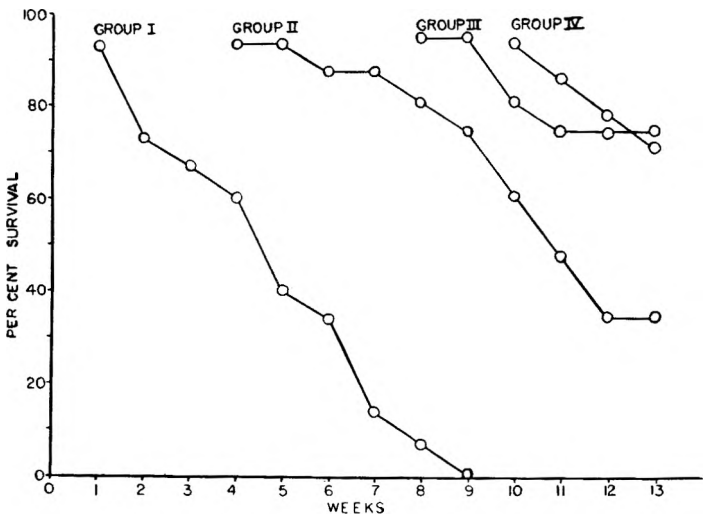

Fig. 1 The percentage survival of male rats during a 10-week period when they were receiving 200 *r* x-irradiation weekly, and for three weeks following the termination of radiation. The following daily dosages of ethyl linoleate were administered: group I, 0 mg; group II, 10 mg; group III, 50 mg; and group IV, 100 mg.

no supplement of this ester; moreover, the curves for survival are progressively higher for each group as the amount of ethyl linoleate administered is increased. The increased protective action of ethyl linoleate against x-irradiation as the dosage is increased is shown in table 2. In this case, the successive periods at which LD_{25}, LD_{50}, and LD_{75} were observed become progressively longer, or are not reached at all, coincident with the higher dosage of ethyl linoleate.

TABLE 2

The survival of male rats receiving a low-fat diet, with or without ethyl linoleate, and subjected to x-irradiation in an amount of 200 r weekly for 10 weeks

CATEGORY	GROUP I	GROUP II	GROUP III	GROUP IV
Body weight at start of radiation, *gm*	205.9	254.0	290.1	287.6
Number of rats per group	15	15	15	13
Ethyl linoleate given daily, *mg*	0	10	50	100
LD (lethal dose) reached, *days* [1]				
25%	28	54	71	86
50%	54	73	..	..
75%	69	..	..	..
Average survival time, *days* [2]	50.7 ± 5.8	71.5 ± 5.7	83.8 ± 3.4	87.4 ± 2.0
M.D.: S.E.M.D. compared with group I [3]	...	2.70	4.93	5.98
Average exposure, *r*	1453	1826	1973	2000
Number of rats alive at end of test (91 days)	0	5	11	9
Per cent survival at 91 days	0	33.3	73.3	69.2

[1] The horizontal bars indicate that deaths did not reach 50% or 75% during the period of observation.

[2] Including the standard error of the mean calculated from formula $\sqrt{\Sigma d^2/(n-1)}\Big/\sqrt{n}$; "d" is the deviation from the mean and "n" represents the number of observations.

[3] When this value exceeds 3.0, the results are considered to be highly significant.

DISCUSSION

The experiments confirm the fact that the protective effect of ethyl linoleate against successive sublethal doses of x-irradiation increases progressively when dosages of 10, 50 and 100 mg daily are given to rats. Thus, the average survival time of rats receiving only 10 mg of ethyl linoleate weekly was 71.5 days, as contrasted with a mean survival time of 50.7 days for the negative control rats. The average length of survival was 83.8 days for group III (50 mg ethyl linoleate daily) and 87.4 days for group IV (100 mg ethyl linoleate daily). The progressive nature of the protective action of increasing dosages of this ester is evident from the comparison of the time at which the LD_{25} was reached. Thus, in the 4 groups, these values were as follows: 28 days (I), 57 days (II), 71 days (III) and 86 days (IV). The LD_{50} was reached only in the control group (I) and the lowest linoleate group (II), while the LD_{75} was reached only in the control group (I). Because of this progressively longer survival of groups II, III and IV, the total average amount of x-irradiation to which these rats were subjected became increasingly greater. In fact, in group IV, in which no fatalities occurred prior to the cessation of irradiation, the average dosage was 2000 *r*, as contrasted with an average value of 1453 *r* in the control group (I). Although the variations in survival time of groups II, III and IV are not sufficient to show statistically significant differences between them, it is quite probable that they would have been significant had the experiments been continued for a longer interval. At the end of 13 weeks, the tests were discontinued; animals still alive at this time were considered to have survived only 91 days.

The ethyl laurate given as a diluent to groups II to IV was without effect on survival, since the dosage was never greater than that given to group I. The lower weight of the control rats at the start of the x-irradiation resulted from the fact that they had previously been used on bioassay tests, as negative controls, while the other groups had

received the same basal diets and the same linoleate dosages as were employed here. It is not believed that variations in survival can be attributed to this difference in starting weights.

The present tests indicate that the optimum linoleate dosage for survival following x-irradiation probably exceeds 100 mg per day in male rats. Thus, it would appear that the optimum quantity of linoleate required for protection against x-irradiation and to produce growth are within the same range. It would also appear, on the basis of the quantitative requirements, that the protective action results from a generalized effect rather than from an effect on a specific organ or tissue.

SUMMARY

The protective effect of linoleate against x-irradiation in the rat has been confirmed.

On the basis of the average length of survival, and from comparison of the periods required for LD_{25}, LD_{50} and LD_{75} to be reached in male rats subjected to x-irradiation, it was demonstrated that the protective effect of ethyl linoleate became greater with increasing dosage.

The optimum dosage for protection against x-irradiation injury, in male rats, probably exceeds 100 mg per day.

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FOREWORD

Concerning Supplements to

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A large part of the cost of most scientific journals is borne by the subscribers. The publication of lengthy manuscripts in THE JOURNAL OF NUTRITION would require a further increase in the subscription price or, alternately, would reduce the number of pages which could be assigned to other authors who desire to publish articles in this Journal. Accordingly, in the interest of our subscribers, it is necessary to limit the number of pages made available to any author and for the presentation of material dealing with any one subject.

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GEORGE R. COWGILL