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THIRTY-SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

MANGANESE DEFICIENCY IN RATS: CONGENITAL NATURE OF ATAXIA [1,2]

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(Received for publication June 18, 1958)

INTRODUCTION

Ataxia in the offspring of manganese-deficient animals was first observed in the chick by Norris and Caskey ('39). These investigators also found that the ataxic chicks were not cured by administration of manganese after hatching, either by injection or by feeding (Caskey and Norris, '40; Caskey et al., '44). Previously, Daniels and Everson ('35) had concluded that the high death rate observed by them as well as by others (Orent and McCollum, '31) in young of manganese-deficient rats was due to a congenital debility. Shils and McCollum ('43) confirmed the report of Daniels and Everson that female rats deficient in this element were capable of raising foster stock or control young, although their own young died, and found also that many of the deficient offspring exhibited an ataxic condition with incoordination and loss of equilibrium.

The ataxic symptoms observed in manganese-deficient young would seem to indicate some dysfunction of the nervous system, due to either (or both) a structural or a biochemical abnormality. Since previous investigators (Shils and McCollum, '43; Caskey et al., '44; Hill et al., '50) were unable to find histological lesions in the brains of ataxic animals, the

[1] This investigation was supported in part by research grant no. A–1340 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

[2] Presented in part at the 22nd annual meeting of the American Institute of Nutrition, April, 1958 (Hurley, Everson and Geiger, '58).

existence of a biochemical abnormality in the nervous system seemed reasonable. Acetylcholinesterase plays an important role in the normal functioning of nervous tissue (Nachmansohn, '40a). Furthermore, it appears to rise in concentration during developmental periods in which there is an increase in motility or muscle coordination or both. Such correlations were indicated by studies of the chick embryo (Nachmansohn, '38a), the sheep embryo (Nachmansohn, '40b), the rat (Nachmansohn, '38b; Metzler and Humm, '51) and the developing embryo of Amblystoma (Sawyer, '43a, b).

It therefore seemed of interest to examine the effect of manganese deficiency upon acetylcholinesterase activity of the brain. Measurements were made in whole brain homogenates of young from manganese-deficient and normal females, from the 19th day of gestation to the 28th day after birth.

The congenital nature of the defect resulting in ataxia was further explored by experiments designed to establish the period during embryonic development which is critical for the occurrence or prevention of ataxia.

EXPERIMENTAL

Animals and diets. The diets were composed primarily of fresh whole homogenized milk, fortified commerically with 400 I.U. of vitamin D per quart. The milk was supplemented with the following nutrients, in micrograms per 100 ml of milk: copper (as copper sulfate) 118, iron (as ferrous sulfate) 120, iodine (as potassium iodide) 4, pyridoxine 100, and corn oil 0.3 ml. For the control groups, manganese (as manganous sulfate) was added in amounts to provide 560 μg for each 100 ml of milk.

A mixture of crystalline vitamins in cerelose, to which was added cod liver oil, was given three times each week, in amounts to provide the following intake in micrograms, per day: calcium pantothenate 500, *p*-aminobenzoic acid and riboflavin, each 100, thiamine hydrochloride, pyridoxine and nicotinic acid, each 300, menadione 250, folic acid 6, biotin 2.5, vitmain B_{12} 0.3, choline 10 mg, inositol 5 mg, alpha-tocopherol

1.1 mg, ascorbic acid 1 mg, vitamin A 150 and vitamin D 15 U.S.P. units each.

Weanling female rats of the Sprague-Dawley strain were purchased from commerical sources and maintained on the milk diets until they reached maturity. They were then housed in individual cages, and the estrous cycles were determined by daily vaginal smear. Normal males, maintained on a stock diet, were used for mating. In one group of experiments, the surviving young born to females on the milk diets were continued on these diets, and also allowed to reproduce.

Judgment of ataxia. In order to judge the presence of ataxia with greater objectivity than is possible when using only visual inspection, an apparatus was designed consisting of a wooden stand holding on end a plywood board, one-half inch thick, with cloth nets on either side. The animal being tested was placed on the one-half-inch surface, and observed as it manipulated itself over the length of the board. Normal animals were able to walk from one end to the other without any difficulty. The severely ataxic animals, however, were unable to walk on the board, and in many cases, could not remain on the surface even momentarily without falling off. Animals with mild cases of ataxia were able to retain their hold on the board for short periods of time, but fell off when they attempted to walk across, or showed difficulty in walking.

Acetylcholinesterase. Brain acetylcholinesterase activity was measured by the titrimetric method of Aprison, Nathan and Himwich ('54). The animals were sacrificed by decapitation, the whole brain including the medulla was removed, weighed rapidly, frozen with dry ice and stored frozen until analyzed. For the fetal samples, the mother was decapitated, the uterus was opened and the embryos were removed quickly, The procedure described above for handling of the brains was then followed.

Critical period during gestation. To establish the period during gestation which was critical for the occurrence of ataxia, manganese-deficient females which had previously given birth to defective young were remated, and maintained

on the deficient ration until the desired day of gestation. They were then transferred to the manganese-supplemented diet, and continued on this ration until their young were weaned. The day on which sperm were found in the vaginal smear was considered to be the first day of gestation. The animals were observed twice during each 24-hour period, and mating was arbitrarily considered to have occurred either at midnight or at noon. Supplementation on the appropriate day was therefore also begun at either of these two hours, so that manganese feeding was always started at the beginning of the stated day of gestation.

TABLE 1

Birth, survival and incidence of ataxia in normal and manganese-deficient rats

DESCRIPTION	NO. OF LITTERS	YOUNG BORN			BIRTH WEIGHT	SURVIVAL TO 28 DAYS	
		Total	per litter	Dead		% of live young	Ataxic
		No.	*No.*	%	*gm*		%
Normal							
First litters	27	208	7.7	4.3	6.3	51.8	0
Second litters	14	130	9.3	1.5	6.3	67.2	0
Second generation	8	71	8.9	9.9	5.8	85.9	0
Manganese-deficient							
First litters	78	586	7.5	11.3	5.8	13.0	66
Second litters	19	162	8.5	4.9	5.8	28.4	76
Second generation	20	133	6.7	9.8	5.8	7.5	100

RESULTS

The effect of manganese deficiency in the maternal diet on the birth, survival and incidence of ataxia in the young is shown in table 1. Results are given for the first and second litters and for young born to second-generation animals. The average number of young born per litter was slightly decreased by the deficiency. It ranged from 7.7 to 9.3 in the normal animals, and from 6.7 to 8.5 in the deficient females. The percentage of young found dead at birth was significantly increased by the manganese deficiency. Only 4.3% of the

young born to manganese-supplemented females in first litters were dead at birth, while 11.3% of the first-litter young of deficient females fell into this category. In second litters, 1.5% of manganese-supplemented young were dead at birth, as compared with 4.9% of deficient young. Young born to females maintained on the milk diets for two generations showed the same proportion of individuals dead at birth (9.8 and 9.9%).

Birth weights were little affected by manganese deficiency. They averaged 5.8 and 6.3 gm for the normal young, and 5.8 gm for the deficient groups.

Survival of the young to 28 days was strikingly affected by the deficiency. Of the living young born to females receiving the manganese-supplemented diet, 52 to 86% survived 28 days. The females receiving the manganese-deficient diet bore young of which only 7.5 to 28% survived to that age.

Manganese deficiency also had a striking effect on the incidence of ataxia in the surviving young. In no case was ataxia observed in the offspring of rats receiving the manganese-supplemented diet. When manganese was withheld from the diet, the incidence of ataxia in the surviving young was 66% in first litters, 76% in second litters and 100% in the surviving young born to females maintained on the deficient diet for the second generation. The ataxic animals exhibited incoordination, lack of equilibrium, head retraction and head tremor.

The results of the assay of acetylcholinesterase activity in whole brain are shown in figure 1. No significant differences between the manganese-supplemented young and the deficient animals were observed. Both groups showed a rising activity from birth to about 21 days of age.

The data derived from experiments undertaken to establish the critical period during gestation for the occurrence of ataxia are summarized in table 2. Animals in which supplementation was begun on the 7th, 10th or 12th days of gestation are grouped together, since there were no differences in their responses. As was expected from the previous work (table 1), manganese deficiency had no effect on the number of young

born per litter. Survival of the young, however, was affected. When supplementation was begun on the 14th day of gestation or before, survival of the young to 28 days was similar to that found previously in control animals (53 and 87% in the supplementation experiment, as compared with 52 to 86% in previous control groups). Supplementation with manganese delayed

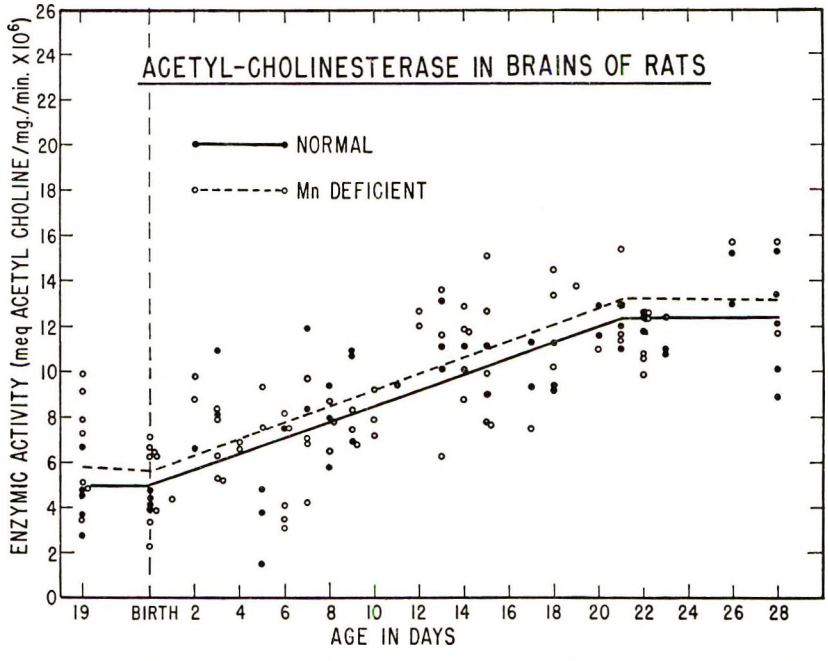

Fig. 1 Acetylcholinesterase in brains of rats. Acetylcholinesterase activity is expressed as milliequivalents of acetylcholine hydrolyzed per milligram of fresh tissue per minute $\times 10^6$.

until the 18th day of gestation resulted in survival of only 26% of live young. When supplementation was begun on the 15th or 16th days of gestation, survival of the young was 36 and 44% respectively.

The time of supplementation, or conversely, the period of deficiency, had pronounced effects on the incidence of ataxia. When supplementation was begun as late as the 14th day, no case of ataxia was seen in the young. When manganese was

withheld until the 18th day of gestation, all of the surviving young were ataxic, and in addition, some young which did not survive to weaning age exhibited this abnormality before they died. When supplementation with manganese was begun on the 15th or 16th days of gestation, about half of the surviving young were mildly ataxic. All of the young born to mothers which received supplementation beginning on the 16th day appeared ataxic shortly after birth, but seemed to show improvement from about the 14th day of age, as judged

TABLE 2

Effect of manganese supplementation at various times during gestation

INITIATION OF SUPPLEMENTATION	NO. OF LITTERS	YOUNG BORN		SURVIVAL TO 28 DAYS	
		Total	per litter	% of live young	Ataxic
		No.	*No.*		*%*
Day of gestation [1]					
7–12	14	105	7.5	53	0
14	6	42	7.0	87	0
15	8	60	7.5	36	48 [2]
16	8	54	6.8	44	46 [2]
18	8	65	8.1	26	100

[1] Day of finding sperm considered first day of gestation.
[2] Mild.

by appearance only before their eyes were open, and by walking on the test board after this time.

Histological examinations made by Dr. Nathan Malamud [3] of the brain, spinal cord, peripheral nerve and skeletal muscle of the hind leg of ataxic animals of two ages, adult and 17 days, and their controls, revealed no differences in the nervous system. A slight inflammatory reaction in the form of interstitial round cell infiltration was observed in the muscle of the 17-day old ataxic animal.

DISCUSSION

The data presented with respect to brain acetylcholinesterase activity do not reveal any effect of manganese defici-

[3] Neuropathologist, Langley Porter Neuropsychiatric Institute, State of California Department of Mental Hygiene, San Francisco.

ency upon this enzyme. At no age, from the 19th day of gestation to 28 days after birth, was there a significant difference in enzyme activity between the young of manganese-deficient females and those of control animals. The ataxic manifestations of manganese deficiency cannot, therefore, be explained by changes in brain cholinesterase, at least under the present conditions. The rise in activity which we observed from birth to the 21st day of age is in general agreement with previous reports (Nachmansohn, '38b; Metzler and Humm '51).

For some time it has been known that liver arginase is decreased in manganese deficiency (Shils and McCollum, '43). More recently, Van Reen and Pearson ('55) have examined the effect of manganese deficiency in the duck on the activity of other liver enzymes. Diphosphopyridine nucleotidase, cytochrome oxidase, catalase and isocitric dehydrogenase activities were not significantly altered by the deficiency. Alkaline phosphatase activity, however, was reduced in kidney, heart and plasma, as well as in liver. It would appear that brain cholinesterase follows the pattern of the liver enzymes investigated by Van Reen and Pearson (with the exception of alkaline phosphatase) in being unaffected by manganese deficiency.

The experiments undertaken to establish the critical period during gestation demonstrate that ataxia in the young could be prevented completely by feeding manganese to the deficient female as late as the 14th day of gestation. Conversely, withholding manganese until the 18th day of gestation resulted in the birth of young which were indistinguishable from those produced by females receiving the deficient diet throughout pregnancy. Despite the fact that manganese supplementation was continued from the 18th day of gestation until the young were weaned, all of the surviving offspring exhibited ataxia at weaning age. These data indicate that manganese deficiency in the pregnant rat produces an irreversible congenital defect, which occurs between the 14th and the 18th days of gestation, and results in ataxia. The response to supplementation begin-

ning on the 15th or 16th days of gestation may mean that the need of the embryo for manganese is critical at that time.

It is of interest to note that the critical period for manganese (at least with respect to ataxia) occurs relatively late in gestation. Most teratologic studies of nutritional deficiencies have indicated that the most susceptible period for production of anomalies in the rat lies within the first two weeks of gestation. Thus, Wilson, Roth and Warkany ('53), in studies of vitamin A deficiency, found that the incidence of defective offspring was substantially reduced by giving vitamin A on the 11th, but not on the 14th day of gestation, while supplementation on the 12th or 13th days decreased the incidence to about half of that observed without therapy. Also, Nelson and her coworkers (Nelson, Wright, Asling and Evans, '55; Nelson, Wright, Baird and Evans, '56) found that the incidence of fetal death or abnormality resulting from a pteroylglutamic acid deficiency in the maternal diet was highest when the vitamin-deficient diet was started on the 9th[4] day of pregnancy. At this time, a 36-hour period of deficiency was sufficient to affect 80% of the embryos. Nelson and her associates (Nelson, Baird, Wright and Evans, '56) also observed that a transitory deficiency of riboflavin in the maternal diet during the 8th to the 14th days of gestation resulted in an incidence of abnormal young as high as that found when the deficient diet was given throughout pregnancy.

Knowledge of the gestational period during which the need for manganese is critical may help to elucidate the role of this element in fetal development. However, the actual nature of the defect resulting in ataxia remains to be discovered. Further studies are in progress.

SUMMARY

Female rats maintained from weaning on a manganese-deficient fresh milk ration were able to bear young not differ-

[4] In discussing the work of Nelson and coworkers, the timing has been changed to correspond with that used in this paper, in which the day of finding sperm is considered to be the first day of gestation, rather than day zero.

ing greatly in number or birth weight from those produced by females receiving a similar diet containing added manganese. Survival of the young was significantly decreased by the deficiency, and most of the surviving deficient young exhibited a pronounced ataxia, with lack of equilibrium, head retraction and tremor.

Analyses of brain acetylcholinesterase activity in the young from the 19th day of gestation to 28 days after birth did not reveal any effect of manganese deficiency upon this enzyme. Both normal and manganese-deficient young showed a rise in brain acetylcholinesterase activity from birth to 21 days of age.

Manganese-deficient females producing defective young were supplemented with manganese at various times during a subsequent pregnancy. Manganese supplementation begun on or before the 14th day of gestation was completely effective in preventing ataxia in the young. When manganese was withheld until the 18th day of gestation (but continued thereafter) survival was low, and all of the surviving young were ataxic. Manganese supplementation begun on the 15th or 16th days of gestation resulted in the birth of young about one-half of which were mildly ataxic.

These data indicate that an irreversible congenital defect resulting in ataxia occurs between the 14th and 18th days of gestation in manganese-deficient female rats.

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EFFECT OF FEEDING LOW LEVELS OF DIETHYLSTILBESTROL ON GESTATION AND LACTATION OF RATS [1,2]

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(Received for publication May 22, 1958)

The feeding of diethylstilbestrol (DES) to beef cattle (Burroughs et al., '55) and lambs (Light et al., '56) increased feed efficiency and rate of gain. Foreman and Porter ('56) and Browning et al. ('57) reported that DES fed to dairy cattle increased persistency of milk production, but Wrenn and Sykes ('57) did not observe that it had this effect. Beeson et al. ('55) found that DES did not improve growth rate and feed efficiency of swine. Sleeth and co-workers ('53) reported similar results using estradiol. Administration of estrogenic materials depressed growth of guinea pigs (Allen and Bern, '42; Wheat et al., '56) and rats (Zondek, '36). Results of the aforementioned investigations indicate that administration of estrogens was beneficial to ruminants but not to simple-stomached animals. Inasmuch as information on the effects of feeding low levels of DES on reproduction and lactation of simple-stomached animals is needed to extend knowledge of the relation of hormones to nutrition, and was not found in the literature, the study reported herein was undertaken.

[1] Contribution no. 569, Department of Chemistry and no. 263, Department of Dairy Husbandry, Kansas Agricultural Experiment Station. These data are to be submitted as a part of a thesis by the senior author to the Graduate School of Kansas State College of Agriculture and Applied Science, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Nutrition.

[2] The authors express their appreciation to H. T. Gier and E. H. Herrick, Department of Zoology, for assistance during this study.

EXPERIMENTAL PROCEDURE

This investigation consisted of two experiments. In experiment 1, 9 female albino rats were divided at random into three groups of three rats each. One group received ground laboratory chow [3] plus 2.2 μg of DES/100 gm body weight/day;[4] another group, ground laboratory chow plus 1.1 μg DES/100 gm body weight/day; and the remaining group (controls), only laboratory chow. The higher of these two levels of DES approximates that used in the recent ruminant nutrition work.

Diethylstilbestrol in a commercial premix [5] was mixed with ground chow so that 10 gm of this food given in the morning would furnish the desired daily intake of DES. Additional food given treated rats was laboratory chow only, and was provided ad libitum in the afternoon after the DES-containing food had been consumed. Rats were fed twice daily, and food intake of each rat was recorded. The supplemented rats received the DES for three days before they were put with males for breeding. DES was not fed during the time of breeding.

The three females of each group were put into a cage with one male. At the end of 5 days, the males were rotated and allowed to remain another 5 days. After this 10-day period, females were placed in individual cages, and DES was added to the food of the treated animals for the remainder of gestation and lactation.

At parturition, each litter was reduced to a uniform size of 10 pups. Litters were weaned at 21 days of age. Since pups were not permitted to eat the chow, their only food was milk.

Total body weight of each litter at weaning was used as the measure of milk production of each respective dam. The

[3] Ralston Purina Company, St. Louis, Mo.

[4] The rats that were fed each experimental level of DES in experiments 1 and 2 were further divided into two sub-groups according to their body weight at the start of the experiment. The sub-groups ranged in weight from 200 to 225 gm and 260 to 300 gm. The average weight of each sub-group was used as the basis for determining the quantity of DES fed to individual rats throughout the experiment.

[5] Supplied by Eli Lilly and Company, Indianapolis, Ind.

total food intake of the dams during gestation and lactation and changes in their body weights were used as further indications of response to DES. These criteria are suitable for indicating the level of milk production in the rat (Folley and Kon, '38).

In experiment 2, 12 female rats were randomly alloted to 4 groups of three rats each. Treatments were the same as in experiment 1, plus a lower level of DES feeding (0.6 μg/100 gm body wt./day) to the additional group. Litters were reduced to 8 pups each instead of 10, and litter weights were recorded at birth, 5, 11, 16 and 21 days of age. Female pups were sacrificed at weaning, and the uteri removed, weighed and fixed for histological study to determine whether estrogens were excreted in the milk of females receiving DES.

RESULTS

Conception. The females did not eat the DES-containing food at a uniform level during the three-day pre-breeding period, and total quantities of DES ingested varied from 0.8 to 6.9 μg. There was no recognizable relationship between quantity of DES ingested and number of days to conception. One female that was in the group receiving the highest level of DES failed to conceive, but several of the same group conceived without apparent difficulty although they had a higher total intake of DES. Fourteen days after separating males and females, two females (one each from groups III and IV, experiment 2) were put back with a male when it was obvious that they were not pregnant. The females had consumed 43 and 80 μg of DES respectively during the 14 days. On pooled data of the two experiments the means and standard errors for number of days to conception were: group I, 4 ± 0.5; group II, 3 ± 0.6; group III, 4 ± 1; and group IV, 4 ± 0.9. The time of conception was considered to be 21 days preceding whelping.

Gestation. The females seemed to detect the DES or the soybean-oil-meal carrier and to find it unpalatable, which per-

haps explains the low consumption of food by some of them. However, after the rats were pregnant they accepted the food-containing DES. One rat continued to refuse the DES food (experiment 1, group IV) and was removed from the experiment.

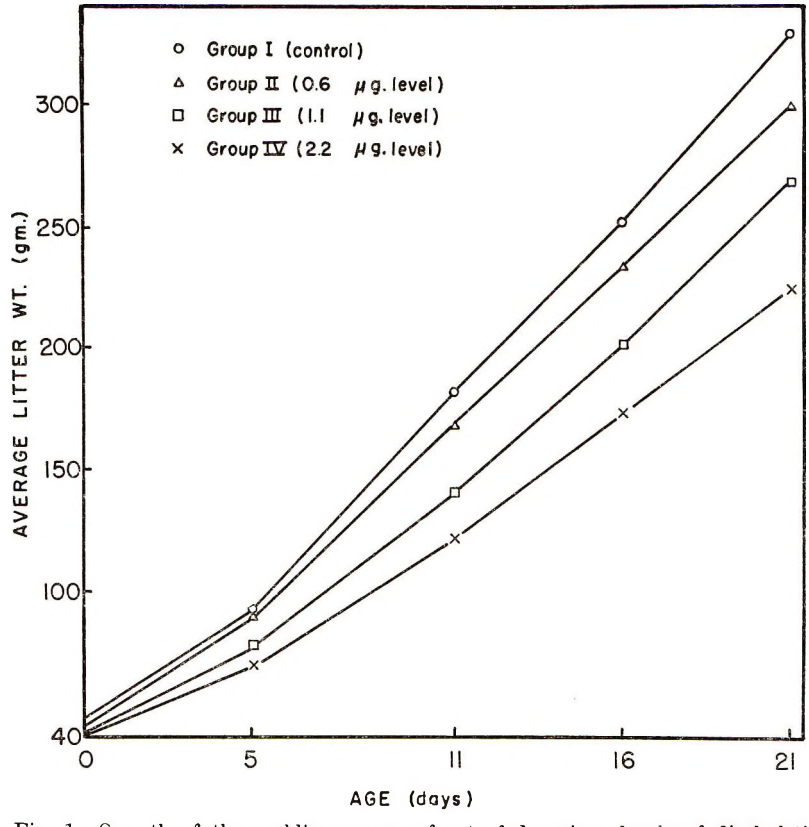

Fig. 1 Growth of the suckling young of rats fed various levels of diethylstilbestrol (experiment 2).

There were no marked effects of DES feeding on gestation. All litters apparently were carried full term. Although the average number of pups per litter of treated rats was similar to that of controls, there was a trend (not significant) toward fewer pups in treated groups, as shown by the following litter size means and standard errors: group I, 14 ± 1.0; group II,

13 ± 1.4; group III, 12 ± 1.0; and group IV, 12 ± 1.3. The most notable effect of feeding of DES during gestation was a decrease in average birth weight of litters from treated rats (fig. 1).

Milk production. There was a marked decrease in lactation of females receiving DES, as determined by the growth of their young. Average weights of pups of each litter at wean-

TABLE 1

Effect of diethylstilbestrol (DES) on milk production in rats as judged by weaning weights of their litters

GROUP NO.	PEN NO.	DES FED DAILY/100 GM BODY WEIGHT	AVERAGE PUP WEIGHT AT WEANING [1]	
			Experiment 1	Experiment 2
		μg	*gm*	*gm*
I	1	0	40.3	41.0
	2	0	40.6	42.2
	3	0	38.0	40.0
II	1	0.6		39.4
	2	0.6		40.4
	3	0.6		37.8
III	1	1.1	35.7	33.4
	2	1.1	36.0	33.3
	3	1.1	31.2	34.8
IV	1	2.2	28.4	29.1
	2	2.2	30.2	27.0
	3	2.2	—	—

[1] Each value in the table represents average pup weight of individual litters. Soon after birth litters were reduced to a uniform size of 10 in experiment 1 and 8 in experiment 2.

ing are shown in table 1. By an analysis of variance, the differences among treatments were found to be significant ($P < 0.01$) in both experiments. There was a significant ($P < 0.01$) linear relationship between weaning weights of suckling rats and level of DES fed their dams in both experiments. Since the linear regression coefficients were not significantly different from each other, the data were pooled and are shown graphically in figure 2. Growth of the 88 nursing pups of experiment 2 is shown in figure 1.

Uterine Weights. Since sex was not considered in reducing litter size, there were differences in the number of female pups per treatment. However, each group of 24 pups had at least 8 females. Uterine weights of the weanling females of experiment 2 are shown in table 2. To adjust for differences

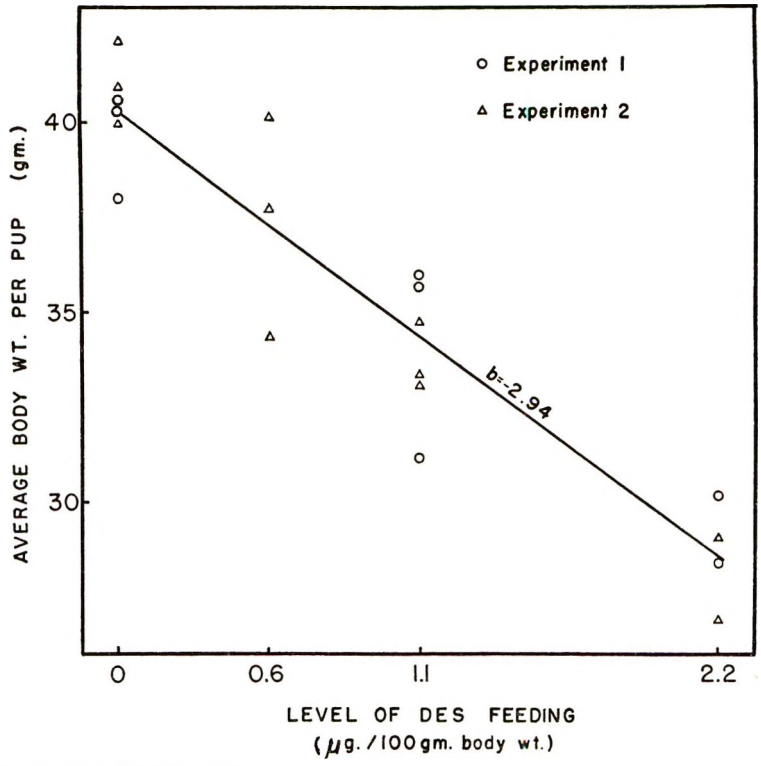

Fig. 2 Relationship between average weight of suckling rats at weaning and the level of diethylstilbestrol (DES) fed to their dams during lactation.

in body weights of pups, uterine ratios, (milligrams of uterus/gm body wt.) $\times$ 100 (Dorfman and Dorfman, '54) also were calculated. Though there was a trend toward heavier uteri (adjusted weights) in weanlings from treated dams, analysis of variance indicated nonsignificance ($P > 0.25$).

To determine if there was estrogenic stimulation of the uteri of weanling females that could not be detected by the

uterine-weight-response method, histological cross-sections of uterine tissue were examined. No differences were observed in uterine tissues of pups from control dams and those receiving DES. For comparison, three weanling females from the control group and two from group IV were fed ad libitum for two days a food containing 0.13 μg of DES/gm. In all 5 rats there was a high degree of uterine stimulation, readily detectable both by uterine-weight-response and histological observation.

TABLE 2

Average uterine weight of weanling young of rats fed diethylstilbestrol (DES), experiment 2

GROUP	NO. OF LACTATING FEMALES	DES FED DAILY/ 100 GM BODY WT. TO LACTATING FEMALES	NO. OF SUCKLING FEMALES	MEAN UTERINE WEIGHT	CORRECTED [1] MEAN UTERINE WEIGHT
		μg		*mg*	
I	3	0	8	22.4 ± 0.8 [2]	55.3 ± 3.6
II	3	0.6	13	21.7 ± 1.0	58.0 ± 4.1
III	3	1.1	11	21.2 ± 1.1	57.8 ± 6.1
IV	2	2.2	8	16.2 ± 0.9	61.6 ± 5.1

[1] Milligrams of uterus/gm of body wt. × 100.

[2] Standard error of mean.

Weight changes and food intake of dams. Data on body weight changes from the start of the study to the end of the lactation period, and food intake during gestation and lactation are summarized in table 3. All 19 rats gained weight, indicating adequate intake of nutrients for milk production. The control animals gained more than the others, but the level of DES administration was not related to changes in body weight.

The control animals consumed more food during gestation (based on the 10 days preceding parturition) and lactation than did rats receiving DES (table 3). Although there appears to be a relationship between food intake and the level of DES fed, differences among groups are less marked when food intake during lactation is adjusted to a body weight basis.

TABLE 3

The effect of feeding diethylstilbestrol (DES) to lactating rats on body weight and food intake [1]

GROUP NO.	NO. OF RATS	DES FED DAILY/ 100 GM BODY WT.	MEAN BODY WT. AT START	MEAN BODY WT. AT END OF LACTATION	MEAN BODY WEIGHT CHANGE	MEAN TOTAL FOOD INTAKE DURING LAST 10 DAYS OF GESTATION	MEAN TOTAL FOOD INTAKE DURING LACTATION	MEAN RATIO OF GRAMS OF FOOD (LACTATION)/100 GM BODY WT. (AV. DURING LACTATION)
		μg	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
I	6	0	229	293	+ 64	232	892	3.09 ± 0.24 [2]
II	3	0.6	272	286	+ 15	166	824	2.90 ± 0.17
III	6	1.1	226	260	+ 34	147	781	3.12 ± 0.32
IV	4	2.2	235	269	+ 34	120	678	2.69 ± 0.18

[1] Pooled data, experiments 1 and 2.

[2] Standard error of mean.

DISCUSSION

The adult rats fed low daily levels of DES for short periods of time conceived and carried their young through a normal gestation. It is possible, however, that the low level feeding of DES caused a partial regression in ovarian function in the rats of this study, as there was a trend (nonsignificant) toward a small number of pups per litter from treated animals. In studies with cattle, Folley and Malpress ('44) and Marshall et al. ('48) reported that high level injections and implantations of DES appeared to cause quiescence of the ovaries.

The lighter birth weights of litters from dams in treated groups possibly can be attributed to the fact that treated dams consumed less food during gestation than did the controls. The reduced food intake of treated rats may have resulted directly from ingestion of small amounts of DES. It is more likely, however, that food intake was reduced because the treated rats were given food without DES only after they had consumed that containing the DES, and, as mentioned previously, most of the rats appeared to dislike this latter food.

Meites ('49) found that as little as 0.001 mg of DES injected daily in young rats caused inhibition of growth due to decreased appetite and food intake. Many workers (Weichert and Kerrigan, '42; Folley and Kon, '38; Edelmann and Gaunt, '41; Meites and Turner, '42; Walker and Matthews, '49) have reported slower rates of growth in the young of lactating rats injected with relatively high levels of estrogenic substances. However, there is disagreement on whether the decrease in growth was due to inhibition of lactation.

Weichert and Kerrigan ('42) reported depressed growth in the young of lactating rats injected with DES, the lowest level used being 0.025 mg daily (about 4 times the highest level fed daily in the present study). They did not think that the slower growth was due to suppression of lactation. The pups appeared to have been subjected to estrogen stimulation, possibly received from the milk. It is possible that the

retardation of growth was due both to a direct action of estrogens on the pups and decreased lactation of the dams. In the present study there was an inverse relationship between the level of DES fed to lactating rats and weight increase in the suckling young. Since no evidence of estrogen stimulation was observed in the suckling young, their smaller weights appear to have resulted from the effect of DES on the lactating female. The lack of agreement in the study of Weichert and Kerrigan, ('42) and the one reported here possibly resulted from differences in levels of DES and method of administration.

Meites and Turner ('42) suggested that a considerable quantity of estrogen might be excreted in milk of lactating rats treated with estrogens and cause inhibition in growth of the suckling young. Excretion of large amounts of estrogens has been reported in the milk of estrogen-injected rats Walker and Stanley, '41), small amounts in milk (Muench, '54) and colostrum (Pope and Roy, '53) of untreated cows, and in the milk of a cow implanted with DES (Lawson et al., '44). However, Lawson et al. ('44) pointed out that a portion of the estrogenic stimulation reported in nurslings (Weichert and Kerrigan, '42; Walker and Stanley, '41) possibly was not from excreted estrogens but from leakage of estrogens from the site of injection. If there were mammary excretion of estrogens in the present study, the quantities were too small to be detected by the rat uterine-weight-response.

Several authors (Folley and Kon, '38; Walker and Matthews, '49; Edelmann and Gaunt, '41) have reported that female rats injected with estrogenic substances lost weight during lactation. Folley and Kon ('38) suggested that at least part of this weight loss resulted from involution of the mammary gland. All lactating rats of the present study increased in body weight; therefore, the lower food intake by the treated animals probably in itself was not sufficient to account for decreased milk production and the consequent

depressed growth of the young. This is further shown (table 3, column 9) by the fact that there was little difference in food intake between the groups treated at the lower and intermediate levels and the control group, after food intake was adjusted to a body weight basis.

SUMMARY

Nineteen adult female albino rats and their litters (168 pups) were used to study some of the effects of feeding low levels of DES (0.6, 1.1, or 2.2 μg/100 gm body weight/day) for three days prior to breeding, and during gestation and lactation.

The feeding of DES did not appear to have a measurable effect on conception or gestation. However, growth of the young of lactating rats receiving DES in their rations was retarded significantly ($P < 0.01$), indicating decreased lactation by females receiving food containing DES. Uteri of weanling females from treated dams were not stimulated, as judged by the uterine-weight-response method, indicating that estrogens were not excreted via the mammary gland in detectable quantities.

Although there was a trend toward decreased total food intake by rats receiving DES during gestation and lactation, food intake apparently was sufficient for lactation, since all lactating females gained weight.

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INFLUENCE OF THE SALT MIXTURE ADDED TO CARIOGENIC DIETS OF COMPARABLE SUCROSE CONTENT

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Despite the use of a synthetic diet similar to that employed in the Harvard laboratories, comparable in sucrose content and in currently recognized essential nutrients, Haldi and Wynn ('52) observed a relatively limited amount of dental caries in the albino rat compared to the extensive lesions previously reported from Harvard by Sognnaes ('48). As the two investigations were conducted with different strains of rats maintained under different laboratory, dietary and genetic conditions, the question arose whether these divergent results might have been due to genetic factors or environmental influences, or to some subtle differences in the purified ingredients of the diets other than the sucrose content.

For these reasons a joint study was conducted by the Emory and Harvard investigators (Wynn, Haldi, Shaw and Sognnaes, '53) in which the two purified diets were fed to littermates of the same strain of rats (Emory-Wistar) under the experimental conditions prevailing at Emory University. The Harvard diet was prepared in the Harvard laboratories by the same methods and from the same supply of components currently in use for routine diet preparation there. The dry components (with the sucrose omitted) and the corn oil containing the fat-soluble vitamins were shipped separately to the

Emory laboratories; there the dry ingredients and the corn oil were mixed in appropriate amounts with the sucrose that was customarily used in the Emory laboratories. A similar experiment was also run with cotton rats. Both experiments confirmed the previous observation that the Harvard diet was more cariogenic than the Emory diet. Therefore it was concluded that the divergent results were at least partially due to an intrinsic difference in the cariogenicity of the Emory and Harvard diets.

Although both diets contained the same total amount (4%) of salt mixture and adequate amounts of all currently recognized essential minerals, there were several seemingly small, but possibly important differences, in the relative amounts of the different minerals in the two diets. The experiments reported in this paper were conducted in the Emory and the Harvard laboratories to determine whether an interchange of the salt mixtures in the two diets would affect their cariogenicity.

PROCEDURE

Emory experiments. The first experiments in this series were initiated some 6 years ago (1952). Weanling rats were taken from the Emory colony of the Wistar strain in tetrad littermate groups and sialoadenectomized by the block dissection technique described elsewhere (Haldi, Wynn, Shaw and Sognnaes, '53). Twenty groups, a total of 80 rats, were selected. The parents had been fed a commercial laboratory chow [1] prior to gestation and during lactation in contradistinction to the Harvard procedure of maintaining the stock colony on the Harvard cariogenic diet. One animal of each tetrad was fed the Emory diet throughout the experimental period of 70 days, one the Harvard diet, one the Emory diet in which the Harvard salt mixture had replaced the Emory salt mixture and one the Harvard diet in which the Emory salt mixture had replaced the Harvard salt mixture. The composition of the salt mixtures is given in table 1.

[1] Purina.

All the animals were kept in individual wire bottom cages and were allowed free access to the experimental diets and to drinking water. At the conclusion of the experiment, the animals were sacrificed, the jaw bones removed, the teeth cleaned thoroughly, examined under a dissecting microscope and scored for dental caries. After a cursory examination in the

TABLE 1

Composition of salt mixtures added to experimental diets

EMORY [1]		HARVARD	
Component	Amount	Component	Amount
	gm		*gm*
$CaCO_3$	850.0	$CaCO_3$	300.0
$MgCO_3$	121.0	K_2HPO_4	470.0
Na_2CO_3	500.0	$CaHPO_4$	680.0
K_2CO_3	706.5	NaCl	670.0
Citric acid	555.5	KCl	115.0
Fe citrate	125.0	Fe citrate	55.0
H_3PO_4 (85%)	765.0	$MgSO_4$ (dried)	100.0
HCl (37%)	267.0	KI	1.6
H_2SO_4	46.0	$MnSO_4$ anhydrous	9.0
KI	0.1	$ZnCl_2$ anhydrous	4.0
$MnSO_4 \cdot H_2O$	13.4	$CuSO_4$ anhydrous	2.4
NaF	0.01	$CoSO_4 \cdot 7H_2O$	0.2
$K_2Al_2(SO_4)) \cdot 24H_2O$	0.16		
$CuSO_4 \cdot 5H_2O$	2.0		
$ZnSO_4 \cdot H_2O$	0.56		

[1] The carbonates, citric acid and citrate were mixed thoroughly. The mineral acids were then added with sufficient water to put the mixture in solution. To this was added the remaining salts that had been dissolved in 100 ml of water. The mixture was then evaporated to dryness over a water bath and then ground to fineness in a corn mill.

Emory laboratory, they were sent to the Harvard laboratory to be examined by a more exacting technique. The values obtained in the cursory and final examination were comparable despite different scoring methods. Because of inconsistencies, to be described later, between the results obtained in experiments conducted in the Emory and Harvard laboratories, an identical experiment was repeated in the Emory laboratory in 1953 and again in 1954.

A 4th experiment with the same procedure was conducted in 1955 with sialoadenectomized rats of the Harvard caries-susceptible strain, representatives of which had been previously sent to the Emory laboratories. This strain had been fed the Harvard diet for several generations in the Harvard laboratories and this same dietary regimen was continued with the growing colony at Emory. Hence both the strain of rats in this experiment and their dietary background were comparable to those used throughout the experiments that were being conducted simultaneously at Harvard. The teeth in this experiment and in the 1953 and 1954 experiments were scored for dental caries in the Emory laboratories only.

Harvard experiments. Two experiments were conducted in the Harvard laboratories with rats of the Harvard caries-susceptible strain. In both experiments the rats were maintained in individual screen bottom cages and provided with tap water and food ad libitum. Close littermate and sex distribution were followed in all experiments. All rats were sialoadenectomized after one stabilization week on the appropriate diet. The animals were sacrificed after a total of 100 days on the experimental regimen and the teeth evaluated for caries by the method of Shaw and others ('44).

In the first experiment one group of animals was fed the Harvard diet containing the Harvard salt mixture and another group the same basic diet in which the Harvard salt mixture was replaced by the Emory salt mixture. In the second experiment the regular Harvard and Emory diets were employed and also the Harvard and Emory diets with salt mixture replacement. There were 4 groups of animals in this experiment. They received the same diets as the rats in the 1952–55 experiments conducted in the Emory laboratories. The Harvard diet with the Harvard salt mixture and the Harvard diet with the Emory salt mixture were prepared in the Harvard laboratories. The Emory salt mixture, the Emory diet with the Emory salt mixture and the Emory diet with the Harvard salt mixture were prepared in the Emory laboratories.

RESULTS

Emory experiments. The Emory diet as shown in table 2 routinely produced a lower average incidence of carious lesions and a lower average caries score in sialoadenectomized rats than the Harvard diet. This observation was true both

TABLE 2

The influence of variations in salt mixture upon the dental caries of sialoadenectomized rats[1] *in the Emory laboratories*

EXP. NO.	DIET	YEAR EXP. CONDUCTED	NUMBER OF ANIMALS	AV. NO. CARIOUS LESIONS	AV. CARIES SCORE
116	Harvard	1952	20	21 (± 1.4)[2]	75 (± 5.0)
117	Harvard	1953	30	26 (± 0.4)	68 (± 4.2)
118	Harvard	1954	20	25 (± 0.4)	66 (± 5.6)
119	Harvard	1955	20	22 (± 0.9)	42 (± 4.1)
		Average		(24)	(65)
116	Harvard + E.S.M.[3]	1952	20	10 (± 1.1)	37 (± 4.0)
117	Harvard + E.S.M.	1953	30	24 (± 0.7)	48 (± 3.6)
118	Harvard + E.S.M.	1954	20	20 (± 1.1)	35 (± 3.1)
119	Harvard + E.S.M.	1955	20	19 (± 1.0)	27 (± 2.1)
		Average		(18)	(37)
116	Emory	1952	20	7 (± 1.1)	22 (± 3.8)
117	Emory	1953	30	18 (± 1.1)	25 (± 2.4)
118	Emory	1954	20	16 (± 1.1)	24 (± 2.7)
119	Emory	1955	20	16 (± 0.6)	19 (± 1.7)
		Average		(14)	(23)
116	Emory + H.S.M.[4]	1952	20	11 (± 0.9)	40 (± 3.8)
117	Emory + H.S.M.	1953	30	25 (± 1.4)	47 (± 2.5)
118	Emory + H.S.M.	1954	20	21 (± 0.7)	30 (± 1.5)
119	Emory + H.S.M.	1955	20	21 (± 0.8)	31 (± 2.4)
		Average		(20)	(37)

[1] The animals in experiments 116 to 118 were the Emory-Wistar strain; those in experiment 119, the Harvard strain.

[2] Figures within parentheses are the standard errors of the means (S.E.M.).

[3] E.S.M. = Emory salt mixture.

[4] H.S.M. = Harvard salt mixture.

for rats of the Emory-Wistar strain and for rats of the Harvard strain. It was also true in another experiment, data of which are not recorded in the table, on intact rats of the Emory strain fed the two diets for 170 days. The difference

in the extent of the carious lesions was manifest almost invariably in littermates fed the two diets and could be readily observed by macroscopic examination.

Quantitatively, the smaller number of lesions in the Emory-Wistar strain rats and the lower caries score on the Emory diet as compared with the Harvard diet were approximately the same in the 1953 and 1954 experiments but the number of lesions was different from those in the 1952 experiment. In the 1953 and 1954 experiments there were respectively 31% fewer carious lesions on the Emory diet as compared with a 67% reduction in the 1952 experiments. There was, however, very little difference in the caries scores in the three experiments.

These quantitative differences in the number of carious lesions in the 1953 and 1954 experiments as compared with those conducted in 1952 may perhaps be accounted for by the fact that in the 1952 experiments the teeth were scored in the Harvard laboratories,[2] whereas the scoring in the 1953 and 1954 experiments was done in the Emory laboratories. The scores for the 1952 experiment are recorded in the table because as stated previously, the teeth were given only a cursory examination in the Emory laboratories before they were sent to Harvard for the final examination. These observations point to the necessity of extreme caution in making quantitative comparisons of evaluations of carious lesions recorded by different observers, especially when different scoring systems were employed.

When the Emory salt mixture replaced the Harvard salt mixture in the Harvard diet, in all 4 experiments there was a lower average incidence of carious lesions and a lower average caries score than when the rats were fed the Harvard diet containing the Harvard salt mixture. Likewise, when the Harvard salt mixture was substituted for the Emory salt mixture in the Emory diet, in all 4 experiments there was a higher average incidence of carious lesions and a

[2] By J.H.S.

higher average caries score than in rats fed the Emory diet containing the Emory salt mixture. However, in both these comparisons, quantitative differences in the results of the experiments can be seen upon close examination of the data in table 2. For example, the Emory salt mixture in the Harvard diet in the 1952 experiment caused a 52% reduction in the average number of carious lesions but only an 8% reduction in the 1953 experiment and an intermediate 20% reduction in the 1954 experiment. At the same time, with respect to the average caries score, the Emory salt mixture in the Harvard diet caused a 51% reduction in the 1952 experiment, a 29% reduction in the 1953 experiment and a 47% reduction in the 1954 experiment. In other words, in the 1952 experiment the Emory salt mixture caused a major decrease in the number of carious lesions but each lesion was the same average size as in the rats fed only the Harvard diet; however, in the 1953 experiment, there was no appreciable decrease in number of lesions but on the average each lesion was appreciably decreased in size. In further contrast, in the 1954 experiment there was both a decrease in the average number of carious lesions and in the average size of each carious lesion. This type of variation in data is difficult, if not impossible, to explain. These variations and those between the Emory experiments described above and the Harvard experiments to be described below strengthen the impression that the above differences were of sufficient importance to merit comment and further investigation.

Harvard experiments. The results of the experiments conducted at Harvard are presented in table 3. In the first experiment replacement of the Harvard salt mixture by the Emory salt mixture in the Harvard diet did not affect its cariogenicity. In the second experiment, however, the rats fed the Harvard diet containing the Emory salt mixture had less tooth decay than the rats fed the Harvard diet containing the Harvard salt mixture. This reduction in tooth decay reached significance at the 5% level. Replacement of the Emory salt mixture by the Harvard salt mixture in the Emory

TABLE 3

The influence of variations in salt mixture upon dental caries of sialoadenectomized albino rats (Harvard Strain) in the Harvard laboratories

EXP. NO.	BASIC DIET	SALT MIXTURE	NO. OF RATS	NUMBER OF CARIOUS TEETH		CR [2]	NUMBER OF CARIOUS LESIONS		CR [2]	CARIES SCORE		
				Av.	SEM [1]		Av.	SEM [1]		Av.	SEM [1]	CR [2]
1	Harvard	Harvard	11	9.0	± 0.8	0.7	16.1	± 1.7	0.6	58.5	± 7.3	0.9
	Harvard	Emory	10	9.7	± 0.5		17.4	± 1.2		66.6	± 5.4	
2	Harvard	Harvard	23	9.3	± 0.4	1.2	18.2	± 1.0	0.3	67.3	± 5.0	0.3
	Emory	Emory	20	10.1	± 0.5	0.9	17.7	± 1.2	2.4	71.4	± 6.8	2.5
	Harvard	Emory	23	8.7	± 0.5	1.5	14.9	± 0.9	0.2	52.1	± 4.4	0.7
	Emory	Harvard	22	10.4	± 0.6		18.6	± 1.4		74.9	± 6.9	

[1] SEM—Standard error of mean.

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

diet, on the other hand, had no effect on the cariogenicity of the diet. Furthermore, no differences were observed in the number of carious lesions or the caries score in the animals fed the Harvard diet containing the Harvard salt mixture and those fed the Emory diet containing the Emory salt mixture.

DISCUSSION

The above results, particularly those from the Emory laboratories, appear to indicate that the Emory and Harvard salt mixtures differ in their influence upon the initiation and progression of dental caries in the laboratory rat.

Evidence has been accumulating in recent years to the effect that the mineral content of a diet is an important factor in determining its cariogenicity. Considerable work along these lines has been reported from the Wisconsin laboratory. In one of the earlier studies (Constant, Phillips and Elvehjem, '52) a 67% sucrose diet was found considerably less cariogenic than a cereal-milk diet. The authors were led to believe that this difference might be due to the dissimilar mineral content of the two diets. Later studies (Constant and others, '54a, b) confirmed the supposition that the cariogenicity of a diet may be related to its mineral components. At the same time Sognnaes and Shaw ('54) reported the results of a long-term study in which the influence on tooth development of the ash of a natural diet was compared with that of a reagent grade salt mixture. Two generations of rats, breeding animals and their offspring, were tested on these salt mixtures. The rats fed a purified ration complete in known essentials developed much more extensive caries than those fed the same ration with the ash of stock diet of whole food as the source of minerals. Other workers have shown that the cariogenicity of a high-sucrose diet may be modified by changing the Ca/P ratio of the diet (Wynn, Haldi, Bentley and Law, '56). The cariogenicity of diets inducing smooth-surface caries has likewise been significantly affected by changing the mineral components of the diet (McClure, Folk and Rust, '56). The

fact must not be overlooked, however, that the differences in mineral concentration between the Harvard and Emory salt mixtures are relatively small in comparison to those in each of the studies discussed above.

While the experiments reported in the present paper indicate that the difference in the cariogenicity of the Emory and Harvard high sucrose diets is related to the difference in the salt mixtures of the two diets, they also suggest that the influence of the salt mixture must be of a sufficiently delicate nature to become easily overwhelmed by a factor or factors that we do not recognize at present. This postulate finds support in (1) the different degree by which the cariogenicity of the Harvard and Emory diets was modified by interchanging the salt mixtures in these diets in the two laboratories; (2) the lack of consistent results in the Harvard laboratories; and (3) the quantitative difference produced by interchange of salt mixtures in experiments conducted in the Emory laboratories at different times. These data serve to indicate the complexity of the dental caries problem; some of the areas of the problem which pose unknown factors; the probable implication of trace mineral factors; and the elusiveness of some of the mechanisms that contribute to the causation or prevention of dental caries.

SUMMARY AND CONCLUSIONS

The difference in cariogenicity of two diets containing comparable amounts of sucrose has been further investigated in a joint study conducted in the Emory and Harvard University laboratories.

The overall results of the experiments indicate that the difference in cariogenicity of the two diets is related in some way to a difference in the salt mixtures customarily added to the diets.

Certain inconsistencies in the results of the various experiments in the two laboratories suggest that the influence of the salt mixtures on the cariogenicity of the diets is of such a subtle nature that it may be overwhelmed by a factor or factors which presently are not recognized.

The data of these experiments emphasize the complexity of the dental caries problem.

ACKNOWLEDGMENT

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ESSENTIAL FATTY ACIDS IN INFANT NUTRITION

I. LINOLEIC ACID REQUIREMENT IN TERMS OF SERUM DI-, TRI- AND TETRAENOIC ACID LEVELS [1]

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Lack of factual data concerning the need for essential fatty acids by human beings prompted a study of the infant's dietary requirement for linoleic acid by correlating the clinical condition of infants with the dietary intake of linoleic acid and with the blood serum levels of unsaturated fatty acids. The significance of assessing the serum levels for the 2-, 3- and 4-double-bond fatty acids in relation to the dietary intake and the requirement for linoleic acid was apparent from the findings in healthy children as compared to the values found in children who were poorly nourished (Wiese, Gibbs and Hansen, '54, Hansen and Wiese, '54). Also, previous studies with dogs disclosed that step-wise increases in the linoleic acid content of the diet were reflected in the blood serum levels of these fatty acids, the appearance of the animals, and the histologic structure of the skin (Hansen and Wiese, '51, Wiese and Hansen, '51).

EXPERIMENTAL

Twenty-one infants under one year of age, 17 of whom were in the hospital for social or minor surgical reasons, were

[1] Supported in part by a U. S. Department of Agriculture contract sponsored by the Human Nutrition Research Division, Agriculture Research Service.

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maintained on different milk mixtures which varied in their linoleic acid content. Four infants were breast fed and remained at home. The hospitalized babies were cared for in a metabolic ward by personnel who were especially oriented for carrying on the study. Each infant was given a milk mixture containing a known amount of linoleic acid for at least one week, then another milk mixture containing a different amount of linoleic acid was given. The diets were fed to the full satisfaction of the infants. Cereals and fruits in measured amounts were given to three subjects. In 19 infants accurate records of the daily food intake were kept and the infants were weighed daily.

Periodic clinical evaluation of each infant was made by the pediatrician in charge. Special attention was given to the occurrence of respiratory tract symptoms, frequency of bowel movements, condition of the skin, and general behavior of the infant.

The basic cow's milk mixtures were skim, half-skim, evaporated, homogenized whole milk and special milk preparations varying in fat and linoleic acid content wherein dietary fats were substituted for the butterfat in cow's milk. Because of the difficulty in varying the linoleic acid in small increments, it was necessary in some instances to supply this unsaturated fatty acid as methyl or ethyl linoleate or trilinolein. The calculated amount of linoleate or trilinolein was measured in a tuberculin syringe and placed directly into the mouth of the infant so as to assure no losses in feeding. To prevent oxidation 0.1% of alpha-tocopherol was added to the esters and triglyceride of linoleic acid. With one exception, each of the artificial milk mixtures provided 66 Cal./100 ml (20 Cal./oz.). When the supplement of linoleic acid was given, carbohydrate in the milk mixtures was decreased in order to keep the Caloric content and percentage of protein constant. Protein was calculated to comprise 15% of the Calories consumed, however, analyses of the milk mixtures showed slight variations in protein content. The proportion of mineral and water did

not vary, inasmuch as the fat and carbohydrate Calories were substituted isocalorically for each other. Iron[3] and vitamins A and D[4] were given daily to all infants whether artificially or breast fed.

The fat, linoleic acid and protein content of the various milks as well as the cereals were determined periodically Total fat and protein were determined by the standard methods of analysis (A.O.A.C., '50). Linoleic acid in the fat extracted from the milk mixtures and cereals was determined by spectrophotometric analysis of the alkali-conjugated fatty acids. The protein, fat and linoleic acid contents of the various milks, milk mixtures and cereals are presented in table 1. The values are expressed both as grams and as percentage of the total Calories. Only the Caloric value of the fruits was considered in the diets because the amounts of protein and fat in fruits were so small.

Blood was drawn after a fast of 8 hours at the end of each dietary period or series of periods. A dietary period was of 7 days' duration. The di-, tri- and tetraenoic acids as well as the total fatty acids were determined in each sample by the method of Wiese and Hansen ('53). At intervals, total serum proteins (albumin and globulin), routine blood counts, and urinalyses were obtained.

In table 2 are presented pertinent data concerning 19 infants. Although the majority of the infants were under three months of age, 4 infants were 3 to 4 months old when first observed. One set of triplets and two sets of twins were studied. The length of time each baby remained on a given diet depended on the availability of the infant for study. Two infants were kept on a particular type of milk for only one dietary period, whereas, one remained on the same regimen for 13 weeks. The period of observation was three weeks for two infants, and for another two infants 26 and 28 weeks. The

[3] Fer-in-Sol ®, ferrous sulfate solution. Mead Johnson and Co., Evansville, Indiana.

[4] Vi-Syneral ®, multi-vitamin preparation. U. S. Vitamin Corp., New York.

TABLE 1

Composition of milk mixtures and cereals

MILK OR CEREAL	PROTEIN	FAT	LINOLEIC ACID	PROTEIN	FAT	LINOLEIC ACID
	gm per 100 ml			*percent of total Calories*		
Skim milk	2.39	0.10	< 0.01	14.5	1.4	< 0.1
+ ethyl linoleate	2.39	0.17	0.07	14.5	2.4	1.0
+ trilinolein	2.39	0.17	0.07	14.5	2.4	1.0
+ methyl linoleate	2.39	0.31	0.21	14.5	4.4	3.0
+ trilinolein	2.39	0.31	0.21	14.5	4.4	3.0
+ ethyl linoleate	2.39	0.45	0.35	14.5	6.4	5.0
Half-skim milk	2.65	1.10	0.03	16.0	15.5	0.5
+ trilinolein	2.65	1.14	0.07	16.0	16.0	1.0
+ ethyl linoleate	2.65	1.17	0.10	16.0	16.5	1.5
+ trilinolein	2.65	1.27	0.21	16.0	18.0	3.0
+ ethyl linoleate	2.65	1.42	0.35	16.0	20.0	5.0
+ ethyl linoleate	2.65	1.63	0.56	16.0	23.0	8.0
Evaporated milk	2.55	2.80	0.06	15.4	39.3	0.9
+ methyl linoleate	2.55	2.87	0.13	15.4	40.3	1.9
+ ethyl linoleate	2.55	3.00	0.27	15.4	42.3	3.9
+ trilinolein	2.55	3.00	0.27	15.4	42.3	3.9
Homogenized milk, whole	3.50	4.20	0.17	20.0	59.1	2.4
Special milks						
A [1]	2.67	3.00	0.21	16.1	42.2	3.0
Compounded [2 3]	2.31	2.05	0.13	15.2	30.5	2.0
Compounded + B [4]	2.31	2.73	0.35	15.2	38.4	4.9
Breast milk [2]	1.22	2.90	0.36	7.6	40.8	5.0
Rice cereal [5]	5.8	4.2	1.43	6.5	11.0	3.7
Barley cereal [5]	10.7	1.6	0.59	12.0	4.1	1.5
Oatmeal cereal [5]	15.7	6.6	2.33	16.9	16.5	5.8

[1] Varamel ®, a form of evaporated milk in which vegetable fats are substituted for butter fat, and which requires the addition of carbohydrate for the usual 20 Cal./oz. formula. The Baker Laboratories, Inc., Cleveland, Ohio.

[2] Infants received cereals and fruits in addition to milk.

[3] Skim and evap. milk, lard and Dextri-maltose.

[4] Lipomul ®, a 40% emulsion of vegetable oils. Upjohn Co., Kalamazoo, Michigan.

[5] Calories, protein, fat and linoleic acid/100 gm cereal.

results represent observations for a total of 184 dietary periods (weeks).

RESULTS

Clinical observations. All 21 infants were healthy and, except for minor effects from surgical procedures (herniorrhaphies), remained so during the course of the study. Mild respiratory infections occurred on 4 occasions. Mild attacks of diarrhea developed in 4 infants when receiving skim milk. The abnormality which appeared to be of some clinical importance was in reference to the condition of the skin. Changes in the skin were observed in 4 infants. The most significant of these was seen on a colored infant (case 6) who was fed the skim milk mixture beginning when she was about 4 months old. After three months, dryness and thickening with fine desquamation of the skin were observed. When her diet was changed to a compounded milk mixture which provided about 2% of the Calories as linoleic acid with 30% of the Calories as fat, within two weeks her skin appeared to be perfectly normal. In another infant (case 17) dryness of the skin also seemed to be related to the low fat diet. One of the triplets (case 7), when on the low-fat diet, developed a slight maculopapular eruption which readily disappeared. One infant (case 16), who received the evaporated milk mixture supplemented with ethyl linoleate to equal 3% of the Calories, developed a slight rash which promptly cleared.

Serum lipid findings. For general orientation, the values found for the infants given three of the milks which are used in infant feeding are summarized in figure 1.

The results show the mean levels of the 2-, 3- and 4-double-bond fatty acids for 14 infants who received skim milk, 12 infants who received evaporated milk, and 4 infants who were fed from the breast. The Caloric intakes of linoleic acid for these three milks were < 0.1, 0.9 and 4.0%, respectively. The serum values are presented both as milligrams per 100 ml as well as percentage of the total fatty acids. It may be noted

TABLE 2

Summary of data on 19 infants under study

CASE	SEX	RACE	STATUS AT BIRTH	TYPE OF MILK [1]	AGE	AV. WT.	AV. GAIN	REMARKS
					Wks.	*Kg*	*gm/day*	
1	M	W	full term	skim	14 to 16	6.90	0	Not fed ad lib. Herniorrhaphy
				evap.	16 to 18	7.05	22	Excellent condition
2	M	W	full term (twin case 3)	evap.	17 to 21	5.40	28	Herniorrhaphy. After surgery slight febrile response, lower intake (95 Cal./kg), slower wt. gain
3	M	W	full term (twin case 2)	skim	17 to 20	3.80	23	Herniorrhaphy. Slight febrile response
				evap.	20 to 21	4.00	11	Lower intake, slower weight gain
4	F	C	premature	skim	3 to 7	2.60	25	Hospitalized for social reasons
				evap.	7 to 11	3.60	27	Hernia discovered, operated at 8 wks. of age. Excellent progress
5	F	C	premature	evap.	6 to 8	2.95	15	Hospitalized — social reasons. Excellent course
				skim	8 to 11	3.60	52	Condition good
6	F	C	full term	skim	16 to 29½	6.10	30	Hospitalized — social reasons. After 2½ wks. crusting lesions on ears and cheeks. At 4 weeks mild diarrhea. At 9 weeks maculo-papular eruption
				special compounded	29½ to 36½	8.70	30	Skin eruption cleared. Condition good
				special comp. + B	36½ to 39	9.50	30	Normal course
7	F	C	full term (triplet cases 8 and 9)	skim	1 to 3½	2.60	42	Hospitalized — social reasons. After 3 weeks, maculo-papular rash which cleared spontaneously. Frequent loose stools
				skim + 1% ML	10 to 14½	4.40	8	Frequent loose stools. No pathogen isolated
8	F	C	full term triplet 7 and 9)	skim	1 to 3½	2.75	45	Hospitalized — social reasons. Frequent loose stools. No pathogens isolated. Oral thrush, 1 mo. old
				evap.	8½ to 14½	4.45	19	Respiratory infection, no complication
9	F	C	full term (triplet 7 and 8)	skim	1 to 3½	2.60	41	Hospitalized — social reasons. Loose stools, no pathogens isolated. At 1 mo., oral thrush, also prickly heat
				skim + 2% MP 1%ML	10 to 12	4.00	lost 3 gm	Frequent loose stools
				evap. + 1% ML	12 to 14½	4.10	15	Respiratory infection, no complication
10	M	C	full term	evap.	4 to 6	4.30	10	Sibling had tuberculosis
				skim	6 to 9	4.45	15	No complications

11	M	C	premature	evap.	8	to 13	3.80	40	Hospitalized — social reasons
				skim	13	to 17	4.70	37	No complications
				skim + 1% EL	17	to 22	5.30	12	No complications
				skim + 5% EL	22	to 25	5.90	25	No complications
12	F	W	full term	breast	8	to 16½	6.25	28	Kept at home. No complications
13	F	W	full term	breast	8	to 22	5.40	15	Kept at home. No complications
14	F	W	full term	half-skim	2	to 7	3.95	40	Hospitalized — social reasons
				half-skim + 1% EL	7	to 12½	4.60	25	No complications
				half-skim + 7½% EL	12½	to 14½	5.10	28	Respiratory infection and diarrhea. Cleared with supportive care
15	F	C	full term	skim	1	to 5	3.75	33	Hospitalized — social reasons. Respiratory infection, no complications
				skim + 5% EL	5	to 10	4.60	28	No complications
				half-skim + 4½% EL	10	to 13	5.40	31	Trichomonas vaginitis. Recovery with treatment
				homogenized whole	13	to 19	6.40	41	No complications
16	M	W	full term	evap.	1	to 3	3.80	40	Hospitalized — social reasons
				evap. + 3% EL	3	to 8	4.25	24	Rash on cheeks — cleared
				evap.	8	to 12	5.20	31	
				skim	12	to 16	5.80	23	
				skim + 1% T	16	to 18	6.10	34	
				skim + 3% T	18	to 20	6.40	12	
				half-skim + 2.5% T	20	to 22	6.70	44	
				skim	22	to 25	7.10	19	
				A	25	to 29	7.70	22	Question of craniostenosis — not proven
17	F	C	premature	evap.	7	to 10	2.50	26	Hospitalized — social reasons
				skim	10	to 12	2.75	12	Seborrhea scalp, weeping lesions in neck. Developed incarcerated hernia — operated successfully
				half-skim + 0.5% T	12	to 14	3.40	55	Respiratory infection — no complications. Excoriation of neck and seborrhea cleared
18	F	C	premature (twin case 19)	skim	3	to 4	2.60	65	Hospitalized — social reasons
				skim + 1% T	4	to 6	2.75	15	
19	F	C	premature (twin case 18)	skim	3	to 4	2.60	74	Hospitalized — social reasons
				skim + 3% T	4	to 6	2.75	15	

[1] Key to symbols: A = Varamel (see footnote 1 in table 1); B = Lipomul (see footnote 4 in table 1); ML = methyl linoleate; EL = ethyl linoleate; MP = methyl palmitate; T = trilinolein.

that the same relationships exist using either method of expression. However, inasmuch as total fatty acids differ among individual infants and at times in the same infant, we prefer to express the values for the unsaturated fatty acids as percentage of the total fatty acids. Even with rather wide differences in total fatty acid content, the distribution of the 2-, 3- and 4-double-bond fatty acids remains remarkably constant under the same dietary conditions.

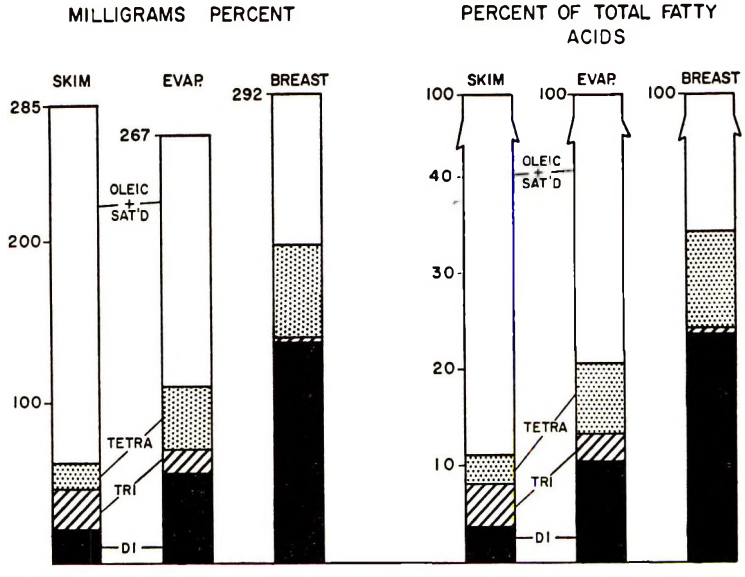

Fig. 1 Serum fatty acids for infants fed skim, evaporated, or breast milk.

Summary data for the serum fatty acids for all the infants arranged in relation to the intake of linoleic acid in step-wise increments are given in table 3. In regard to the infants who were maintained on the *skim milk* mixture for periods varying from one to 13 weeks in duration, the mean values for dienoic acid were very low, as were those for the tetraenoic acid, whereas the trienoic acid values were high compared with serum levels for infants who received evaporated milk. In spite of the fact that *half-skim milk* supplied only 0.5% of the Calories as linoleic acid with a total fat of 15% of the Cal-

TABLE 3

Serum fatty acids in relation to stepwise increases in intake of linoleic acid

LINOLEIC	FAT	MILK [1]	NUMBER OF INFANTS	AV. NO. WKS.	TFA	SERUM UNSATURATED FA [2]		
						di-	tri-	tetra-
% of total Cal.					*mg %*			
< 0.1	1.4	skim	14	3.2	285	3.6	4.5	2.9
0.5	15.5	½ skim	1	6.7	277	6.7	4.0	4.7
0.9	39.3	evap.	13	2.9	267	10.5	2.7	7.4
1.0	2.4	skim + EL	2	3.5	319	6.1	5.9	2.3
1.0	2.4	skim + T	2	2.0	280	11.6	4.1	6.6
1.0	16.0	½ skim + T	1	2.0	236	13.5	4.2	7.0
1.5	16.5	½ skim + EL	1	5.0	282	10.2	3.4	3.2
1.7	30.1	special comp.	1	7.0	329	22.1	3.7	6.5
1.9	40.3	evap. + ML	1	2.0	264	16.3	4.7	5.5
2.4	59.1	homog. cow	1	6.0	242	17.4	2.0	8.4
3.0	42.2	A	1	4.0	271	30.3	2.5	8.1
3.0	4.4	skim + ML	1	4.0	240	10.8	4.8	7.4
3.0	4.4	skim + T	2	2.0	253	17.9	3.5	9.7
3.0	18.0	½ skim + T	1	2.0	298	19.3	2.8	10.1
3.9	42.3	evap. + EL	1	4.0	313	20.7	1.7	9.0
4.0	40.8	breast	4	9.0	292	23.7	0.6	10.0
4.6	42.5	sp. comp. + B	1	2.0	218	22.8	3.8	6.0
5.0	6.4	skim + EL	2	3.5	283	12.6	5.0	4.9
5.0	20.0	½ skim + EL	1	3.0	308	17.9	3.2	7.4
8.0	23.0	½ skim + EL	1	1.5	206	18.9	1.9	5.7

[1] Key to symbols: EL = ethyl linoleate; ML = methyl linoleate; T = trilinolein; A = Varamel (see footnote 1 in table 1); B = Lipomul (see footnote 4 in table 1).

[2] As percentage of total fatty acids.

ories, there was a slight, but definite, increase in the di- and tetraenoic acids with little change in the trienoic acid levels of the serum compared with values for skim milk. When *evaporated milk* was given, the di- and tetraenoic acids were definitely higher while the trienoic acid level was lower than for the infants who received skim or half-skim milk. Supplementation of skim and half-skim milk with linoleic acid at a 1% Caloric intake (approximating that of evaporated milk) resulted in serum levels for di- and tetraenoic acids similar to those for evaporated milk only when the supplement was given as trilinolein. Linoleates were much less effective in altering the di- and tetraenoic acids of the serum than was the triglyceride form of linoleic acid even at the 1.5% Caloric level. Increasing the linoleic acid intake to 1.9% of the Calories by supplementation of evaporated milk with methyl linoleate, likewise, resulted in a somewhat lower serum level for dienoic acid and a higher level for trienoic acid than when the linoleic acid was provided in its natural form at Caloric intakes of 1.7 to 2.4%. At higher Caloric intakes of linoleic acid in general, the dienoic acid of the serum continued to increase when each type of milk (skim, half-skim, evaporated) was supplemented with linoleic acid. However, the tetraenoic acid level did not go above a maximum of 11.4% of the total fatty acids which was the value observed for one breast fed infant. Changes in the tri- and tetraenoic acid levels with the administration of linoleic acid were slower and less uniform than for dienoic acid. In other words, the serum level for dienoic acid reflected to a greater degree the intake of linoleic acid than did the levels for the tri- and tetraenoic acids. At all levels of intake the ester form of linoleic acid was more effective in increasing the serum content of di- and tetraenoic acids when given together with natural fat (as in half-skim or evaporated milk) than when given with skim milk, but at no level of intake did the response equal that of trilinolein. These differences are demonstrated graphically in figure 2. The intake of fat alone, irrespective of the amount

of linoleic acid, showed no consistent correlation with the dienoic acid content of the serum.

Statistical comparison of the serum unsaturated fatty acids in relation to the intake of linoleic acid was possible for three types of milk (skim, evaporated and breast). These data are given in table 4. No distinct differences were observed in the mean levels of the total fatty acids; however, significant differences were found for the di-, tri- and tetraenoic acids in the

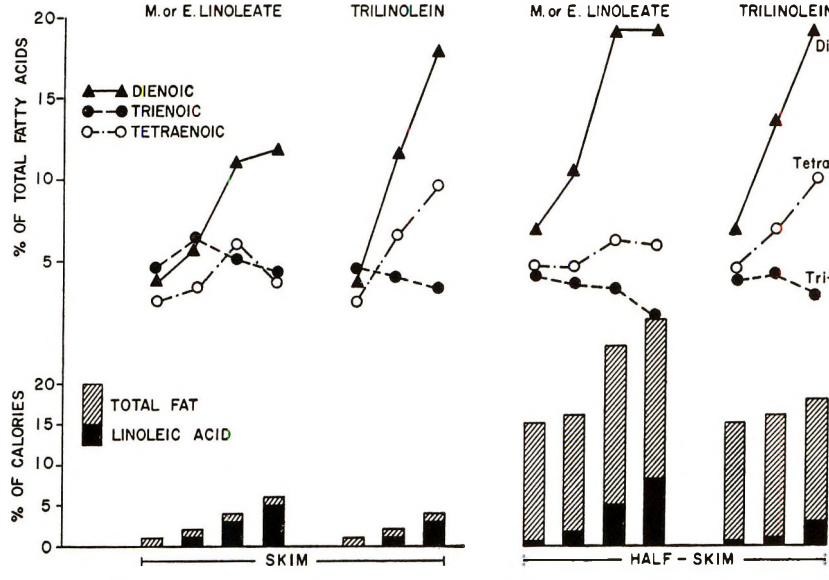

Fig. 2 Serum unsaturated fatty acids in relation to form of linoleic acid in diet.

serum of infants given skim, evaporated and breast milk. It may be noted that the serum level for dienoic acid in infants who received breast milk is more than twice the level for those infants who received evaporated milk. The values for tetraenoic acid are higher also in the breast fed group than in the evaporated milk group, but the percentage difference is not as great as for dienoic acid. The trienoic acid level which varies inversely with the linoleic acid content of the diet was found to be extremely low in the breast fed infants.

TABLE 4

Statistical summary of unsaturated fatty acids of serum for infants who received skim, evaporated and breast milk

MILK	NUMBER		TFA	DI-	TRI-	TETRAENOIC
	Infants	Determinations				
			mg %	*% of TFA*	*% of TFA*	*% of TFA*
Skim	14	15				
Mean			285	3.6	4.5	2.9
SD			57.77	1.36	1.14	1.31
SE_m			15.44	0.36	0.30	0.35
Evap.	13	14				
Mean			267	10.5	2.7	7.4
SD			39.30	1.25	0.82	2.39
SE_m			10.90	0.36	0.22	0.66
P[1]			> 0.10	< 0.001	< 0.001	< 0.001
Skim	14	15				
Mean			285	3.6	4.5	2.9
SD			57.77	1.36	1.14	1.31
SE_m			15.44	0.36	0.30	0.35
Breast	4	9				
Mean			292	23.7	0.6	10.0
SD			46.07	1.84	0.24	1.15
SE_m			16.29	0.65	0.08	0.40
P			> 0.10	< 0.001	< 0.001	< 0.001
Evap.	13	14				
Mean			267	10.5	2.7	7.4
SD			39.30	1.25	0.82	2.39
SE_m			10.90	0.36	0.22	0.66
Breast	4	9				
Mean			292	23.7	0.6	10.0
SD			46.07	1.84	0.24	1.15
SE_m			16.29	0.65	0.08	0.40
P			> 0.05	< 0.001	< 0.001	< 0.001

[1] ''P'' was derived from calculated ''t'' values (when $N < 30$) using a two tail test of the one tail probability table in Dixon and Massey, '51.

Other laboratory findings. The amount of linoleic acid in the diet was found to have no effect on the total serum protein, albumin-globulin ratio, hemoglobin, erythrocyte, leukocyte or differential white cell counts. No abnormal urinary findings which could be attributed to the diet were noted in any of the infants.

DISCUSSION

Inspection of the data presented may lead one to ask whether or not it is possible to establish normal values for the unsaturated fatty acids in the blood serum of infants. Also, is it possible to delineate *minimum, normal* and *optimum* levels for the di-, tri- and tetraenoic acid of the serum in terms of the intake of linoleic acid?

It must be acknowledged from clinical experience that, in general, the use of evaporated milk mixtures has been successful in infant feeding. In other words, when the intake of linoleic acid comprises about 1% of the Calories in infants under one year of age, the clinical condition usually is satisfactory and one may assume that serum levels found for these infants are adequate and may be considered as *normal.* In our series, the mean values for the 2-, 3- and 4-double-bond fatty acids were found to be 10.5, 2.7 and 7.4% of the total fatty acids, respectively. However, are these levels minimum or optimum for healthy infants? In spite of the great advances made in the artificial feeding of infants, no milk has proven to be superior to that of human milk. The linoleic acid in breast milk comprises from 4 to 5% of the Calories and uniformly the blood serum levels for the di-, tri- and tetraenoic acids have been found to be about 23, 1.0 and 10% of the total fatty acids, respectively. It would seem possible that the superior quality of breast milk, in part, might be attributable to its high linoleic acid content with the consequent high levels for the 2- and 4- and a low level for the 3-double-bond fatty acids in the serum. Early findings on the low incidence of skin manifestations in breast fed infants may be of some significance in this regard. Grulee and Sanford ('36) found that eczema was 7 times more frequent in infants receiving cow's milk mixtures than in breast fed infants, and twice as common in infants given both a cow's milk mixture and mother's milk as in those who were maintained entirely on the breast. Robinson ('40) found skin eruptions twice as frequent in artificially fed as in breast fed infants.

Suggestive skin changes were observed when three of our infants were on skim milk. Of particular interest were the findings in the baby who was maintained on the diet low in fat for 13 weeks. In the light of current studies (Hansen et al., '57) it seems likely that the skin changes while on the skim milk would have been more severe if this infant had been younger (< three months of age). Also, if the young infants (one to 4 weeks of age) had been maintained on skim milk for longer periods than under the conditions of our study, skin changes might have been more prevalent. Even though clinical signs of a deficiency of essential fatty acids were not evident in all the babies given a diet low in fat (skim milk), the blood serum of all of these infants showed significantly less di- and tetraenoic acid and more trienoic acid than the serum of those receiving evaporated or breast milk. Decreases in the 2- and 4-double-bond fatty acids were observed even at the end of one week on skim milk (cases 10, 15, 18, 19) and were particularly low in young infants under one month of age (cases 7, 8, 9, 15, 18, 19). It would seem justifiable to conclude from our observations that the subjects who received skim milk did not have serum levels for unsaturated fatty acids equivalent to those found in healthy infants. Furthermore, the normal serum levels for the di-, tri- and tetraenoic acids in healthy infants under one year of age who were given evaporated milk mixtures would be considered *minimum normal* levels. However, it was observed that these levels were not always maintained when the linoleic acid intake was 1% of the Calories. It was only when linoleic acid was provided in the form of a glyceride, either in a natural fat or as trilinolein that the serum levels for the di-, tri- and tetraenoic acids were in the range observed for babies fed evaporated milk mixtures.

Although difficult to prove by clinical observation, optimum levels for the di-, tri- and tetraenoic acids may well be those found in breast fed infants. In this connection it may be noted that the dienoic acid level in breast fed infants was

increased over that of the evaporated milk group relatively more than was the tetraenoic acid. It appears that the tetraenoic acid content of serum remains in a maximum range of 10 to 12% of the total fatty acids even with high intakes of linoleic acid and high serum levels of dienoic acid. The levels previously observed for healthy children 4 to 15 years of age were in this same range for tetraenoic acid, but were somewhat higher for dienoic acid than breast fed infants (Wiese, Gibbs and Hansen, '54).

Further evidence in support of the adequacy of a *minimum* of 1% Caloric intake of linoleic acid and an *optimum* of 4% linoleic acid for infants under one year of age is gained from observations made on Caloric consumption and from skin manifestations in another group of infants, the results of which will be presented in subsequent reports.

SUMMARY AND CONCLUSIONS

Clinical observations and chemical determinations of the serum levels for the di-, tri- and tetraenoic acids in relation to the dietary intake of linoleic acid have been made on 21 infants under one year of age. The results appear to justify the conclusion that the dietary requirement for linoleic acid in healthy infants may be evaluated in terms of the blood serum levels for the di-, tri- and tetraenoic acids. As determined by alkaline conjugation of the total fatty acids and spectrographic analysis of the soaps, values $< 5.0\%$ of the total fatty acids for di- and tetraenoic acids and $> 5.0\%$ for trienoic acid are suggestive of a dietary deficiency of linoleic acid. *Minimum normal* levels for the di-, tri- and tetraenoic acids appear to be 10.5 ± 1.3, 2.7 ± 0.8 and $7.4 \pm 2.4\%$ of the total fatty acids, respectively. These values result when the dietary intake of linoleic acid in the form of a glyceride constitutes about 1% of the total Calories. *Optimum* levels for the di-, tri- and tetraenoic acids in the serum of healthy infants may well be 23.7 ± 1.8, 0.6 ± 0.2 and $10.0 \pm 1.1\%$ of the total fatty acids, respectively. These values are attained

in breast fed infants in whom the linoleic acid intake is about 4% of the total Calories. Further data to be presented regarding Caloric consumption and clinical observations pertaining to the skin support the conclusions.

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EFFECT OF FOLIC ACID DEFICIENCY ON MYOGLOBIN CONCENTRATION IN SKELETAL MUSCLE OF THE GUINEA PIG [1,2]

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Myoglobin is a respiratory pigment which acts physiologically as a short-time oxygen store in the muscle cell (Millikan, '39). It contains the same prosthetic heme group as does hemoglobin. Despite its basic physiologic role, little is known concerning its synthesis, and the influence of hemopoietic factors on its formation is almost totally unexplored. In view of the position of folic acid in hemopoiesis, it might be expected to influence the formation of myoglobin.

METHODS

Male Connaught strain guinea pigs, weighing from 220 to 390 gm were paired in increasing order of weight, caged individually, and maintained in air-conditioned quarters. The experiment was repeated three times (A, B, C). In experiments A, B and C (table 1) there were initially 6, 11 and 15 pairs of animals respectively. One member of each pair was

[1] The material presented in this paper is taken from a thesis submitted by Willard R. Faulkner in partial fulfillment of the requirements for the degree of Doctor of Philosophy from Vanderbilt University.

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selected at random and fed a purified folic acid-deficient diet similar to that reported by Woodruff et al. ('53) but which contained no *p*-aminobenzoic acid; the other member served as the pair-fed control. To the deficient ration was added 1% of sulfasuxidine as a means of aggravating the folic acid deficiency. Controls received a similar diet containing 2 mg/kg of folic acid but no sulfasuxidine. Although sulfasuxidine might conceivably influence myoglobin concentration, there is no evidence to suggest that it would account for the data here reported. Just prior to feeding, 100 gm of diet were moistened with 10 gm of water to prevent scattering and thereby improve the pair-feeding. Each animal received each day orally 5 mg of ascorbic acid in aqueous solution. Water was given ad libitum since control of water intake does not predictably alter the distribution of water between body compartments.

Hematologic examinations were performed on blood from the ear. Erythrocyte and leucocyte counts were made by standard techniques and the packed cell volume (PCV) by the micro method of Guest and Siler ('34). Hemoglobin was determined by the oxyhemoglobin method of Evelyn.

At the time of sacrifice, the combined gastrocnemius and soleus muscles of both legs were removed for analysis. Myoglobin was determined on these muscles by the method of Poel ('49).

RESULTS

Animals were sacrificed in pairs when leukopenia and anemia were pronounced in the deficient member of the pair, but before he was morbid. It was difficult to determine the exact state of the animal and in many cases death ensued before the experiment was terminated. In the three experiments, 4, 7 and 4 pairs survived at the end of 34, 40 and 37 days respectively. At that time a definite deficiency, as judged by anemia and leukopenia, was present for each deficient animal. The anemia was predominantly of the normochromic-normocytic type (table 1). Mean body weights of the animals in both deficient and control groups in all three experi-

TABLE 1

Mean terminal values in guinea pigs on folic acid-deficient diet as compared to controls

	EXPERIMENT NUMBER					
	A (4) [1]		B (7) [1]		C (4) [1]	
	Deficient	Control	Deficient	Control	Deficient	Control
Erythrocytes (RBC), cells/mm^3 × 10^6	4.50 ± 0.33	5.71 ± 0.24	4.58 ± 0.27	6.55 ± 0.85	4.75 ± 0.90	5.25 ± 0.08
Hemoglobin (Hb), gm/100 ml	11.6 ± 0.8	14.6 ± 0.8	12.2 ± 0.6	15.4 ± 0.5	11.1 ± 0.1	13.9 ± 0.0
Packed cell volume, (PCV), per cent	37 ± 3	45 ± 2	37 ± 1	49 ± 2	37 ± 1	45 ± 1
Leukocytes, cells/mm^3 × 10^3	2.80 ± 0.40	7.10 ± 1.90	2.20 ± 0.30	10.80 ± 2.30	2.50 ± 0.40	11.00 ± 2.00
MCV [2]	82	79	81	75	78	86
MCH [3]	25	26	27	24	23	26
MCHC [4]	31	32	33	31	30	31
Myoglobin, mg/gm	1.41 ± 0.28	1.05 ± 0.12	1.64 ± 0.15	1.12 ± 0.11	1.80 ± 0.29	1.42 ± 0.09

[1] Values in parentheses are number of surviving pairs of rats. Values = Mean ± S.E.

[2] Mean corpuscular volume (MCV) in cubic microns = $\frac{\text{Volume of packed red cells (PCV), ml per 100 ml} \times 10}{\text{Red cell count (RBC), million per mm}^3}$.

[3] Mean corpuscular hemoglobin (MCH) in micromicrograms = $\frac{\text{Hemoglobin, gm per 100 ml} \times 10}{\text{Red cell count (RBC), million per mm}^3}$.

[4] Mean corpuscular hemoglobin concentration (MCHC) in per cent = $\frac{\text{Hemoglobin, gm per 100 ml} \times 100}{\text{Volume packed red cells (PCV), ml per 100 ml}}$.

ments paralleled each other closely throughout the feeding period.

The folic acid-deficient guinea pigs had a higher concentration of myoglobin in their muscles than did the pair-fed mates (table 1). The water content of the muscle from control and experimental animals was the same. Therefore, one can conclude that the differences in myoglobin concentration were not a reflection of an altered state of hydration of the muscle.

DISCUSSION

Folic acid deficiency limits hemoglobin formation as is evidenced by a reduced hemoglobin in humans and animals chronically deficient in folic acid and by the increase in hemoglobin which occurs following administration of folic acid to deficient animals. The precise mechanism of this limitation has never been defined.

The synthesis of serine from glycine and formate (Deodhar et al., '55; Kisliuk and Sakami, '55; Alexander and Greenburg, '55), as well as in the breakdown of serine to glycine (Elwyn and Sprinson, '50; Braunshtein and Vilenkina, '51; Blakley, '54) are influenced by folic acid. Much evidence supports that of Shemin and Wittenberg ('51), who established the biosynthetic incorporation of glycine into the porphyrin nucleus. Totter and his associates ('47, '49) have presented indirect evidence indicating that folic acid is involved in the synthesis of porphyrins. In addition, glycine is utilized in the synthesis of the globin moieties of myoglobin and hemoglobin (Rossi-Fanelli et al., '54). In view of these relationships, it is especially striking that the occurrence of folic acid deficiency anemia in the guinea pig is not associated with a decrease in the myoglobin concentration.

The unexpected finding of an increased concentration of myoglobin in folic acid-deficient guinea pigs indicates some fundamental difference in the metabolism of blood hemoglobin and myoglobin. Present knowledge does not permit one to state whether this difference is one of rate of degradation of

the heme moiety of hemoglobin and myoglobin or an unsuspected basic difference in the synthetic mechanism for heme or protein in the two pigments. It would be surprising if folic acid were not essential for myoglobin formation. If this were the case, however, in folic acid deficiency the intermediates unused in hemoglobin synthesis might be present in unusual amounts and thereby stimulate myoglobin formation. The metabolism of these heme-containing pigments must be studied further before one can account for the observed difference in effect of folic acid deficiency on these two closely similar pigments.

SUMMARY

In guinea pigs there occurred an unexpected increase in myoglobin content in the skeletal muscle of folic acid-deficient animals compared to pair-fed controls. In this species, the change in myoglobin was associated with a typical folic acid deficiency anemia and alteration in the hematologic picture. Possible implications of this finding for the existence of important differences in the synthesis or rate of degradation of myoglobin and hemoglobin are discussed.

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INFLUENCE OF AMINO ACID DEFICIENCIES AND PROTEIN LEVEL ON THE PLASMA CHOLESTEROL OF THE CHICK[1]

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Much attention has been focused recently on dietary factors involved in the regulation of the plasma cholesterol level. Low-protein diets have produced elevated plasma cholesterol values in the rat (Moyer et al., '56) and chicken (Kokatnur et al., '58; Nishida et al., '58). Normal plasma cholesterol values were obtained when normal protein levels were fed. Jones and Huffman ('56) have reported an elevation in plasma cholesterol when the protein level of the diet was either below or above a limited range of values.

The present studies were undertaken to elucidate the function of protein with special reference to the role of certain amino acids in influencing the blood cholesterol level.

EXPERIMENTAL

The breed, sex, age and number of chicks used in each experiment are indicated in each table of results. The chicks were reared in electrically heated batteries and the experimental rations and water were supplied ad libitum. The nonprotein portion of the diets used, together with an outline

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Poultry Science, New Brunswick. Supported in part by grants-in-aid from the Nutrition Foundation and the U. S. Public Health Service.

of the experiments to be discussed, is presented in table 1. No attempt was made to balance the diets calorically because this would have required the use of fat as an additional variable. Amino acid supplementation was made at a constant

TABLE 1

Composition of the non-protein portion of the diets and experimental plan

INGREDIENT	AMOUNT
	%
Mineral mix [1]	5.34
Fiber	3.00
Corn oil	3.00
Choline Chloride	0.20
Vitamin mix [1]	0.15
Vitamin A, D and E concentrate [1]	0.10
Glucose (cerelose)	to 100

[1] Fisher and Johnson ('56).

Experimental plan

EXP.	TABLE	PROTEIN		AMINO ACID UNDER STUDY	OTHER VARIABLES
		Source	Levels		
			%		
1	2	Isolated soybean [1]	10, 25, 40	Methionine	None
2	3	Soybean meal	15	Methionine	Heating
3	4	Casein	10, 25, 40	Arginine, methionine	None
4		Isolated soybean, casein, gelatin	25, 75	None	None
5	6	Sesame meal	15, 25	Lysine	None
6	6	Hydrolyzed casein	10, 25	Tryptophan	None
7	7	Isolated soybean	7, 13, 19, 25, 31	None	Cholesterol

[1] Drackett Assay Protein C–1.

percentage of the protein. All additions to the diets were made at the expense of glucose (cerelose).

Chicks were fed their designated diets for three-week periods (unless otherwise indicated) during which time body weight and feed consumption were recorded at weekly intervals. Since the feed records reflect only the growth data they are omitted from the tables of results for sake of greater

clarity. Plasma cholesterol was determined at the termination of the experiment by the method of Zlatkis et al. ('53).

RESULTS

The first experiment in this series involved the feeding of three levels of isolated soybean protein [2] with and without methionine supplementation. Although separate groups of male and female chicks were employed, only pooled average values are presented in table 2, since there was no significant sex difference in the blood cholesterol levels. The effect of

TABLE 2

The effect of protein level and methionine supplementation on the plasma cholesterol and weight gain of the young chick [1]

ISOLATED SOYBEAN PROTEIN	METHIONINE SUPPLEMENTATION	WEIGHT GAIN [2]	PLASMA CHOLESTEROL [2]
%	%	*gm*	*mg %*
10	—	55	371
10	0.12	91	262
25	—	154	226
25	0.31	162	230
40	—	190	178
40	0.50	198	158

[1] Crossbred Columbian ♀ × NH ♂ chicks were started at one day of age; one group of 7 males and one group of 7 females were placed on each dietary regime.

[2] Standard deviation ($\sqrt{}$ error mean square from analysis of variance): gains 33 gm, plasma cholesterol 79 mg%.

protein level is highly significant ($P < 0.001$) and the supplementation with methionine produced a small but significant ($P < 0.05$) decrease in plasma cholesterol. The response to methionine was obtained primarily at the lowest level of protein.

Experiment 2 was designed to test protein quality at a suboptimal protein level for the young chick (15%) in terms of the plasma cholesterol response. Unheated soybean meal was compared to properly heated meal with the results as indicated in table 3. The higher plasma cholesterol on the un-

[2] Drackett Assay Protein C–1, The Drackett Products Company, Cincinatti, Ohio.

heated meal coincides with the poorer growth rates and suggests that both methionine supplementation and heating provide more available protein for the growing birds.

Since the plasma cholesterol level in the first two studies seemed negatively correlated with the growth rate, an attempt was made in the next series of experiments to ascertain whether the protein and methionine effects were specific or merely a reflection of the overall protein nutrition of the growing animal. Experiment 3 was designed to study the specific effect of methionine in such a manner that the plasma cholesterol response would not be confounded by a growth stimulation.

TABLE 3

The influence of protein quality on the plasma cholesterol and weight gains of the young chick [1]

PROTEIN SOURCE	METHIONINE SUPPLEMENTATION	WEIGHT GAIN	PLASMA CHOLESTEROL
	%	*gm*	*mg %*
15% raw soybean [2]	—	60 ± 14 [3]	464 ± 44 [3]
15% raw soybean	0.2	156 ± 20	392 ± 19
15% heated soybean	—	155 ± 12	310 ± 40
15% heated soybean	0.2	225 ± 14	276 ± 10

[1] Day-old Vantress male chicks were started at one day of age with 10 chicks per lot.

[2] Both the raw and the heated soybean meal contained 50% protein (N × 6.25) and were therefore added at the 30% level to provide 15% protein.

[3] Standard error of the mean.

Casein had been shown by Fisher et al. ('56) to be only marginally deficient in sulfur amino acids for the chick when supplied at the 20% level. It was therefore reasoned that with casein as the protein any specific methionine effect on the plasma cholesterol level might be obtained independently of an added growth response from methionine. In addition, arginine was studied as another variable since casein is severely deficient in arginine for the chick. The design and results of this experiment are given in table 4. Using casein as the protein source, it appears that protein and methionine have separate effects on plasma cholesterol not necessarily re-

lated to growth rate. The analyses of variance for both the cholesterol data and weight gains are presented in table 5. It can be seen that the contribution of methionine to weight gains at the low protein level made the supplementation of methionine significant at the 0.05% level. Inspection of the growth data reveals that the highest protein level, supplemented with arginine and methionine, did not promote better

TABLE 4

The effect of varying the level of casein and of supplementation with arginine and methionine on the plasma cholesterol and weight gain of the growing chick [1]

CASEIN PROTEIN	AMINO ACID SUPPLEMENTATION [2]	WEIGHT GAIN [3]	PLASMA CHOLESTEROL [3]
%		*gm*	*mg %*
10	— — —	40	350
10	0.4% arginine	133	256
10	0.07% methionine	57	302
10	0.4% arginine + 0.07% methionine	292	226
25	— — —	82	232
25	1.0% arginine	369	204
25	0.18% methionine	113	236
25	1.0% arginine + 0.18% methionine	359	187
40	— — —	333	166
40	1.6% arginine	355	186
40	0.28% methionine	306	164
40	1.6% arginine + 0.28% methionine	361	144

[1] Vantress male chicks, 10 per lot were placed on experiment at 7 days of age.

[2] Glycine was added to all groups at the rate of 2% of the protein level.

[3] Standard deviation ($\sqrt{\text{error mean square}}$ from analysis of variance table 5): weight gain 68 gm, plasma cholesterol 37 mg%.

growth than the intermediate or 25% protein level equally supplemented. On the other hand plasma cholesterol was depressed by the higher protein level. Arginine appeared to exert its influence on both cholesterol level and growth by increasing the amount of protein available to the chick. This is best illustrated by the contribution of the interaction terms in the analysis of variance. In both cases the arginine $\times$ protein level contribution is considerably larger than the methionine $\times$ protein level contribution.

To obtain further information on the role of protein in relating growth rate and plasma cholesterol level, a 75% protein diet (isolated soybean, casein and gelatin) was compared in experiment 4 to a standard 25% protein diet (isolated soybean protein). The weight gains and plasma

TABLE 5

Analysis of variance of the plasma cholesterol (upper part of table) and weight gain data (lower part) as given in table 4

SOURCE	DEGREES OF FREEDOM	MEAN SQUARE	F	
Protein level	2	141,932	103.75	$P < 0.001$
Arginine	1	50,718	37.07	$P < 0.001$
Methionine	1	15,120	11.05	$P < 0.01$
Arginine × protein level	2	18,024	13.18	$P < 0.001$
Methionine × protein level	2	2,576	1.88	
Arginine × methionine	1	1,491	1.09	
Protein level × arginine × methionine	2	2,180	1.59	
Error	107 [1]	1,368		
Total	119			
Protein level	2	433,275	94.72	$P < 0.001$
Arginine	1	734,611	160.60	$P < 0.001$
Methionine	1	26,196	5.73	$P < 0.05$
Arginine × protein level	2	130,196	28.46	$P < 0.001$
Methionine × protein level	2	26,815	5.86	$P < 0.05$
Arginine × methionine	1	15,075	3.30	
Protein level × arginine × methionine	2	20,943	4.58	$P < 0.05$
Error	107 [1]	4,574		
Total	119			

[1] One degree of freedom lost for missing value.

cholesterol levels of week-old cockerels maintained on experiment for 10 days were 113 gm and 130 mg% for the 25% protein diet; 92 gm and 89 mg% for the 75% protein diet. The cholesterol lowering effect of the high-protein diet was highly significant ($P < 0.01$) although there was no significant difference in weight gains ($P > 0.20$).

Experiments 5 and 6 furnished further evidence indicating that there is no generalized relationship between plasma cholesterol and growth rate, but rather that different amino acids behave independently in influencing or not influencing the former. As shown in table 6, lysine and tryptophan were the amino acid variables and sesame meal and hydrolyzed casein the respective deficient proteins. With both of these amino acids no cholesterolemic response was obtained at

TABLE 6

Effect of lysine and tryptophan on the plasma cholesterol and weight gain of the young chick [1]

PROTEIN SOURCE	AMINO ACID SUPPLEMENTATION	WEIGHT GAIN	PLASMA CHOLESTEROL
		gm	*mg %*
15% sesame	—	53 ± 4 [2]	204 ± 22 [2]
15% sesame	0.24% lysine	133 ± 5	141 ± 11
25% sesame	—	129 ± 7	180 ± 11
25% sesame	0.40% lysine	209 ± 7	152 ± 7
10% hydrolyzed casein	0.07% tryptophan	37 ± 7	234 ± 10
10% hydrolyzed casein	0.10% tryptophan	15 ± 3	202 ± 14
25% hydrolyzed casein	0.13% tryptophan	96 ± 10	178 ± 6
25% hydrolyzed casein	0.17% tryptophan	186 ± 12	195 ± 13

[1] Crossbred Columbian ♀ × NH ♂ chicks were used in this study. Eight chicks per lot, started at one day of age were used for the sesame experiment, and 10 chicks per lot, placed on the experimental diets at 7 days of age were used for the hydrolyzed casein experiment.

[2] Standard error of the mean.

the 25% level of protein whereas highly significant ($P < 0.001$) growth responses were evident.

A final experiment was conducted (experiment 7) to compare the effect of a varied protein level [3] in birds supplied with or without an exogenous source of cholesterol. The data in table 7 indicate that with high-protein levels essentially "normal" blood cholesterol values are obtained in the presence or absence of dietary cholesterol. Thus, a diet well balanced in protein and amino acids provides almost complete protection against the hypercholesteremia induced by incorporating 2% cholesterol into the diet.

[3] See footnote 2, page 369.

TABLE 7

Effect of protein level and cholesterol supplementation on the plasma cholesterol of the young chick [1]

ISOLATED SOYBEAN PROTEIN [2]	CHOLESTEROL SUPPLEMENTATION	WEIGHT GAIN	PLASMA CHOLESTEROL
%	%	*gm*	*mg %*
7	—	20 ± 8 [3]	253 ± 14 [3]
7	2	56 ± 27	611 ± 104
13	—	315 ± 10	199 ± 10
13	2	316 ± 8	342 ± 29
19	—	487 ± 22	156 ± 4
19	2	503 ± 15	185 ± 10
25	—	551 ± 17	139 ± 3
25	2	562 ± 7	169 ± 5
31	—	595 ± 9	143 ± 3
31	2	537 ± 14	165 ± 11

[1] Ten crossbred Columbian ♀ × NH ♂ chicks were used per lot. Chicks were reared on a practical starting ration for 4 weeks before being placed on the experimental diet.

[2] Drackett Assay Protein C-1. Methionine was added to all diets at the rate of 1% of the protein supplied.

[3] Standard error of the mean.

DISCUSSION

The data presented in this report indicate that proper protein and amino acid nutrition is of importance in the regulation of the circulating cholesterol, both of endogenous and exogenous origin.

It would appear from these data that it is very difficult or impossible to meet the day-old chick's requirement for protein in terms of maintaining a low plasma cholesterol level. Because of this difficulty and in order to overcome the confounding effects of increased growth with supernormal levels of protein, older chicks were used in the later experiments of this series. When one or three-week old chicks were used in place of day-old chicks the variability in the plasma cholesterol values was also sharply reduced.[4] Experiment 4 indicates that the plasma cholesterol of the chick can be

[4] In comparing the variability of the data from one-day-old chicks (table 3) with those of 7-day-old chicks (table 4) it should be noted that the standard *error* of the mean is listed in table 3 and the standard *deviation* in table 4.

lowered to the level of that in the rat by feeding a very high protein diet. This observation indicates that certain amino acids may exert specific effects on the plasma cholesterol level.

At levels of protein which are suboptimal for the maintenance and growth requirement of the chick, amino acid supplementation of deficient proteins appears to make more protein available and thus account for the relationship between an increased growth rate and a decreased blood cholesterol level. However, at optimal or supernormal protein intake levels the 4 amino acids studied behaved in one of three ways: (1) arginine, by making up the deficiency in that amino acid (for casein) thus providing more protein for growth and simultaneously lowering the plasma cholesterol level; (2) methionine, by exerting a specific cholesterol-lowering effect not related to improved growth; and (3) lysine and tryptophan, by correcting deficiencies in these amino acids and improving protein quality and therefore growth but exerting no effect on the plasma cholesterol level.

It is important to stress that the hypercholesteremia in the studies discussed above was not induced by an exogenous source. On the other hand when cholesterol was fed in a low-protein diet, the hypercholesteremia was greatly exaggerated. It might be concluded, therefore, that amino balance and protein nutrition of the animal play an important role in the regulatory mechanism of circulating cholesterol.

SUMMARY

Deficiencies in the amino acids arginine, lysine, methionine and tryptophan were studied at different levels of protein intake in relationship to growth rate and plasma cholesterol of the young chick. It was shown that at suboptimal protein intakes, a hypercholesteremia resulted which could be modified by supplementing the deficient protein with amino acids such that more protein would become available to the bird. At optimal or supernormal protein intakes arginine, when

added to a casein diet deficient in this amino acid, continued to extert a cholesterol-lowering effect which could be explained on the basis of greater protein availability; lysine and tryptophan, when added to proteins deficient in these amino acids, exerted no effect on the plasma cholesterol level and methionine produced a cholesterol-lowering effect which was not related to any improvement in protein quality or growth rate. Although the feeding of cholesterol increased the hypercholesteremia of birds on low-protein diets, essentially normal levels of plasma cholesterol were observed when the protein deficiency was alleviated.

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FILICIDAL CANNIBALISM BY FEMALE ALBINO RATS

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INTRODUCTION

Cannibalism by nursing mothers of newborn rats has probably been noted by most laboratories which breed albino rats for experimental purposes. However, little experimentation has been reported concerning this phenomenon or its alleviation.

In this laboratory, an increase has been noted in the incidence of cannibalism among females fed a modified [1] Bills ('31) breeding diet. This behavior usually takes place one to three days after parturition and rarely occurs after the animals are over three days old. A survey of environmental factors failed to disclose any explanation, and an insufficient supply of some nutrient in the diet was suspected. The Bills diet may be deficient or limiting in certain metabolites necessary for reproductive efficiency and proper maintenance. Data by Hubbell ('54) showed that the reproductive efficiency of rats on the Bills diet was inferior to that obtained when a more complete stock ration was fed. Jaffe ('52) reported that the addition of vitamin B_{12} to the diet improved reproductive performance, and also noted an increase in the weaning weights of the young. Lepkovsky et al. ('51) observed that rats maintained on a vitamin B_{12}-deficient diet showed a decrease in the number of conceptions and an increase in the number of resorptions.

[1] Modified by the addition of 0.015% $MnSO_4 \cdot H_2O$ and 120 gm wheat germ per 4 kg of feed.

Animals reared on the Bills diet in this laboratory are bred exclusively to produce young which are used in the bioassay for vitamin D according to the A.O.A.C. method ('55). Because of the limited information available concerning filicidal cannibalism among albino rats, especially among those on the Bills diet, and also because of the diminished number of weanling rats available for test purposes, investigation of a method for its alleviation seemed desirable.

EXPERIMENTAL

Weanling female albino rats of the Osborne-Mendel strain developed at this Station were placed on either the modified [2] Bills diet ('31) or on one of two test diets. These contained either one or 3% of whole liver powder [3] added to the Bills diet. Litter mates were randomly assigned so that at least one received the control diet and one each the test diets. Food and water were offered ad libitum. These animals were maintained on their respective diets until the termination of the experiment. Animals from each group were mated at 120 ± 10 days. Males were randomly assigned for each mating, one per three females, and received the same diet as the females with which they were mated. An attempt was made to procure at least three litters from each female, different males being used in most instances. Animals were discarded from the experiment only if they failed to conceive twice in succession or became ill. Litters were reduced in size to 10 if more young were cast, and whenever possible a 1:1 ratio of females to males was saved. Offspring of all matings were weaned at 21 ± 1 days and kept on the same diet as the mother until placed on the A.O.A.C. rachitogenic diet No. 2 (A.O.A.C., '55). All weaning weights were corrected by ± 2.5 gm per day to 21 days when necessary. Females were not mated again until at least 21 ± 3 days had elapsed since

[2] See footnote 1, page 377.

[3] Nutritional Biochemicals Corp., Cleveland, Ohio. Material for a preliminary study was supplied through the courtesy of Dr. C. E. Graham, Wilson Laboratories, Chicago, Illinois.

weaning of the previous litter. Offspring of all groups, after being on the rachitogenic diet the required length of time, were assigned at random to the A.O.A.C. line test bioassay for vitamin D (A.O.A.C., '55).

RESULTS

Cannibalism by nursing mothers fed a modified Bills diet can be controlled by the addition of 3% of whole liver powder. There was no difference between the animals of the control group and those receiving the 1% liver supplement. However, there was a highly significant difference between the data obtained at the 3% level and the control as well as between the results at the 3% and 1% levels. These results are summarized in table 1.

TABLE 1

Control of cannibalism by addition of whole liver powder[1] *to the Bills diet*

DIET	NO. OF LITTERS	NO. OF LITTERS EATEN	% EATEN
Control	70	24	34.3
1% liver supplement	49	17	34.7
3% liver supplement	40	5	12.5

Comparing 3% and 1% liver, $\chi^2 = 3.54$, $P = 0.03$ (one-tail test)
Comparing 3% and control $\chi^2 = 5.15$, $P = 0.013$ (one-tail test)

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.

The supplementation of the Bills diet with liver powder has had certain other beneficial effects. The weaning weights of progeny from mothers who received the supplemented diet were significantly higher than those of the controls. This is shown in table 2.

Another factor which had to be considered was whether the addition of 3% of whole liver powder to the breeding diet of the mothers would in any way affect the usefulness of their progeny in the vitamin D bioassay. If the line test was affected, then the modified diet would be useless as a breeding diet for this purpose. As previously noted, progeny from

TABLE 2

Mean weaning weights of progeny from mothers fed Bills diet supplemented with liver powder

DIET	NO. OF LITTERS	AV. WEANING WTS.
		gm
Control	46	38.0 ± 0.92 [1]
1% liver supplement	32	42.6 ± 1.10
3% liver supplement	33	44.9 ± 1.09

Analysis of Variance

COMPARISONS	D.F.	S.S.	M.S.	F.
1% vs. 3% liver	1	88.4297	88.4297	2.28
Control vs. treated	1	908.3126	908.3126	23.37 [2]
Within groups	108	4196.8401	38.8596	
Total	110	5193.5824		

[1] Standard error.
[2] Highly significant.

TABLE 3

Line test scores of animals given U.S.P. vitamin D_2 in doses sufficient to obtain a theoretical line test score of 1.00

SOURCE OF PROGENY	NO. OF ANIMALS	AV. LINE SCORE
Mothers fed 1% liver	22	1.08
Mothers fed 3% liver	22	0.97
Mothers fed control diet	23	1.02

Analysis of Variance

COMPARISONS	D.F.	S.S.	M.S.	F.
Between treatments	2	0.1420	0.0710	1.42
Within treatments	64	3.1994	0.0500	
Totals	66	3.3414		
			P = 0.20 (not significant)	

such mothers were randomly assigned to the vitamin D bioassay. The results of such tests are recorded in table 3. There was no significant difference between the line scores of any group individually, or in the aggregate.

DISCUSSION

Filicidal cannibalism by nursing mothers poses a twofold problem. First, the number of animals which become available

for experimental purposes is greatly diminished, and second, the cause of this action must eventually be ascertained so that it can be controlled. The problem has been of concern in this laboratory since large numbers of animals are needed for the routine assay of vitamin D. In order to obtain satisfactory animals for this test, the breeding diet must be limiting in certain nutrients. Otherwise, progeny from such matings fail to show satisfactory development of rickets. Any changes made in this breeding diet must be carefully considered so that progeny will be suitable for the testing of vitamin D.

Although no work has been reported which directly bears on this cannibalistic phenomenon, investigations noted in the introduction have suggested that the addition of whole liver powder to the Bills diet might alleviate the situation. The addition of liver to the diet has, in effect, resulted in a sharp decline of cannibalism and an increase in the weaning weights of the progeny. Although the 1% liver supplement did not curb cannibalism, the addition of 3% was effective. The reason for the lack of control at the 1% level may be that the intake of the unknown factor which controls cannibalism was insufficient. The nature of this unknown factor is left for subsequent investigation.

The significance of the large increase in weaning weight provided by the addition of liver to the diet will be fully appreciated by investigators who breed albino rats for use in vitamin D bioassays. The body weight of a weanling rat must exceed 44 gm in order for it to be depleted satisfactorily when placed on a rachitogenic diet (A.O.A.C., '55). Accordingly, if the weight is at the specified level at weaning time, the animals can be placed on the rachitogenic diet without delay. As has been shown, the addition of liver to the diet of the mothers does not impair the usefulness of their progeny for the line test for vitamin D. In this connection, it may be noted that Nakayama and Sahashi ('52) have reported no difference in the severity of rickets when rats on a rachitogenic diet were given vitamin B_{12} either orally or intramuscularly.

SUMMARY

The effect of adding whole liver powder to the Bills breeding diet in order to control filicidal cannibalism was investigated. Three per cent of liver significantly lowered the incidence of this action; a 1% level was insufficient.

The addition of either one or 3% of whole liver powder to the mothers' diet significantly increased the weaning weights of the progeny.

Data are presented which show that progeny from such mothers, when used in the line test bioassay for vitamin D, do not adversely affect the results of this assay.

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GROWTH OF THIAMINE-DEFICIENT RATS FED SORBITOL OR ANTIBIOTICS IN RATIONS OF VARYING FAT CONTENT [1]

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Recently Yudkin and Morgan ('57) reported that rats grew well for long periods of time in the absence of dietary B vitamins when 10 or 20% of sorbitol was added to the diet. Most of their diets were devoid of thiamine, and very high in fat and protein. The tissues from the rats fed sorbitol contained substantially more thiamine than those not fed sorbitol, and hence an increase in thiamine synthesis was assumed analogous to that thought to take place when vitamins are "spared" by the presence of appropriate antibiotics in the diets. The present study represents an attempt to confirm the findings of Yudkin and Morgan and to determine whether antibiotics either enhance or diminish the response to sorbitol in diets varying in carbohydrate content but lacking in thiamine. The effect of antibiotics in diets of varying carbohydrate content was also studied.

EXPERIMENTAL

Male weanling rats of the Holtzman or Rolfsmeyer [3] strains, 40 to 55 gm in weight, were divided into groups of 5 each, and

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the rats were housed individually on screens and given food and water ad libitum. The dietary ingredients, in gram quantities, were vitamin test casein 24, salts IV (Hegsted et al., '41) 4, corn oil 4, sucrose 63, and choline chloride 0.1; in milligram quantities, α-tocopherol 3, riboflavin 0.3, pyridoxine O.2, menadione 1.0, nicotinic acid 2.5, calcium pantothenate 2.0, inositol 10.0, biotin 0.01, folic acid 0.2 and vitamin B_{12} 0.002. Vitamins A and D were administered weekly by dropper. These ingredients remained constant in all groups except that in some experiments lard was substituted for sucrose on an isocaloric basis to maintain a constant calorie to protein ratio, the levels of sucrose in the diets being 0, 8.6, 10, and 63 gm. Thiamine was added to certain groups at either a suboptimal level (75 μg per 100 gm control equivalent) or at a level sufficient for good growth. Antibiotics were added at the level of 5 mg per 100 gm control equivalent both with and without sorbitol.[4] In series I 10 gm of sorbitol replaced its caloric equivalent in lard and sucrose (assuming complete utilization of sorbitol); in the remaining series sorbitol was added without caloric compensation.

After 5 weeks the experiments were terminated and the per cent effectiveness of sorbitol or antibiotic was calculated from the weights of the animals at 21 days from the expression:

$$\frac{\text{Average weight gained by groups fed antibiotic or sorbitol} - \text{Average weight gained by control}}{\text{Average weight gained by control}} \times 100$$

It was necessary to use the 21-day data because of the poor survival of the control rats on the thiamine-deficient diets; with certain other exceptions, figure 1, the effects of the various dietary supplements were qualitatively very similar at 35 and 21 days.

[4] Sorbitol was purchased from Distillation Products, Rochester, New York and Atlas Powder Company, New Castle, Delaware. The apparent thiamine content as determined by the thiochrome method was 0.5 μg per 100 gm sorbitol which would represent a maximum of 0.5 μg thiamine added per kilogram of sorbitol-containing diet.

TABLE 1

Increased growth of rats fed penicillin in thiamine-deficient rations

LINE NO.	SERIES	SUCROSE	THIAMINE	SORBITOL	BODY WEIGHT AT 21 DAYS [1]		INCREASE DUE TO PENICILLIN (21 DAYS)	BODY WEIGHT AT 35 DAYS	
					No penicillin	Plus penicillin		No penicillin	Plus penicillin
		gm	*μg*	*gm*	*gm*	*gm*	%	*gm*	*gm*
1	V	—	—	—	95	130	72	93	153
2	V	—	—	10	140	151	12	214	237
3	V	—	75	—	167	173	5	237	238
4	VI	—	—	—	99	112	23	89	147
5	VI	—	—	10	135	137	2	178	210
6	I	8.6	—	10	148	143	— 5	208	231
7	I	10	—	—	76	100	89	--	133
8	II	10	—	—	76	96	80	--	118
9	II	10	—	10	140	146	7	100	216
10	III	10	300	—	173	177	3	261	264
11	III	10	300	10	160	157	— 3	242	240
12	IV	63	—	—	68	91	128	--	77
13	IV	63	—	10	124	128	5	116	132

[1] The average starting weights of the 5 rats in each group were: Series I, 49 gm; Series II, 51 gm; Series III, 50 gm; Series IV, 50 gm; Series V, 46 gm; and Series VI, 45 gm.

TABLE 2

Increased growth of rats fed sorbitol in thiamine-deficient rations

LINE NO.	SERIES	SUCROSE	THIAMINE	ANTIBIOTIC AT 5 MG LEVEL	BODY WEIGHT AT 21 DAYS [1]		INCREASE DUE TO SORBITOL (21 DAYS)	BODY WEIGHT AT 35 DAYS [2]	
					No Sorbitol	Plus Sorbitol		No Sorbitol	Plus Sorbitol
		gm	*μg*		*gm*	*gm*	%	*gm*	*gm*
1	V	—	—	—	95	140	92	93	214
2	VI	—	—	—	99	135	66	89	178
3	V	—	—	Penicillin	130	151	25	153	237
4	VI	—	—	Penicillin	112	137	38	147	210
5	VI	—	—	Aureomycin	103	127	42	97	211
6	VI	—	—	Chloromycetin	86	133	113	76	119
7	VI	—	—	Streptomycin	97	139	85	93	162
8	VI	—	—	Bacitracin	108	141	52	117	210
9	I	8.6 [3]	—	—	76	148	267	—	209
10	II	10	—	—	76	140	256	—	100
11	I	8.6 [3]	—	Penicillin	100	143	84	138	244
12	II	10	—	Penicillin	96	146	111	118	216
13	I	10	75	—	117	158	60	94 (2)	256
14	II	10	75	—	120	154	49	105	149
15	III	63	—	—	68	124	301	—	116
16	III	63	—	Penicillin	91	128	90	77 (4)	132
17	III	10	300	—	173	160	— 15	261	242
18	III	10	300	Penicillin	177	157	— 5.9	264	240
19	V	—	—	Aureomycin	99			88	
20	V	—	—	*p*-Aminophenyl arsanilic acid	100			104	

[1] The average starting weights of the 5 rats in each group were: series I, 49 gm; series II, 51 gm; series III, 50 gm; series IV, 50 gm; series V, 46 gm; series VI, 45 gm.

[2] — indicates no survivors and ( ) indicates the number of rats surviving.

[3] Sorbitol substituted calorically at the expense of both sucrose and fat.

RESULTS

Data presented in tables 1 and 2 illustrate the growth-promoting effects of penicillin and of sorbitol in thiamine-deficient diets, and data from additional antibiotics are presented in table 2. Data for the 5-week interval are included, since the inability of sorbitol to promote continual growth did not become evident until that time.

Dietary sucrose. In agreement with others the growth rate of the rats fed the unsupplemented low-thiamine diets depended upon the percentage of calories furnished as sucrose or fat (table 1, lines 1, 7, and 12). Greater body weights were realized more or less progressively as the level of sucrose in the diet was decreased. Changes in sucrose did not, however, appear to affect survival until sucrose was eliminated entirely, when survival was increased from an average of 28.3 days to over 35 days.

Antibiotics. Dietary penicillin consistently stimulated growth when thiamine was lacking or was present at a limiting level in the diet. The per cent stimulation due to penicillin at 21 days increased as the dietary carbohydrate increased (table 1, lines 1, 4, 7, 8, and 12). No effect of penicillin was noted when the diets contained adequate thiamine (table 1, lines 3 and 10).

On the high-fat diet devoid of thiamine and sorbitol, aureomycin, unlike penicillin, caused no growth stimulation either at a 21 or 35 day interval in one experiment (table 2, lines 1 *vs.* 19) and only a marginal response in another (lines 2 *vs.* 5). Under these conditions chloromycetin appeared to depress growth, bacitracin stimulated growth slightly while *p*-aminophenyl arsanilic acid and streptomycin were without effect.

Sorbitol. The presence of sorbitol in diets lacking or limiting in thiamine consistently stimulated the growth of rats at all levels of dietary sucrose tested (table 2, lines 1, 2, 9, 10, and 15), the response to sorbitol being greater than that to penicillin at equivalent sucrose levels. Growth stimulation due to sorbitol was reduced by moderate levels of thiamine

and was absent when the diet contained adequate thiamine (table 2, lines 13, 14, 17, and 18). In agreement with Yudkin and Morgan ('57) sorbitol stimulated growth in our thiamine-free diet, but the effect did not persist as long as reported under their conditions. In the complete absence of thiamine and antibiotics, sorbitol stimulated growth for 21 days on a high level of carbohydrate (63%) and for more than 35 days

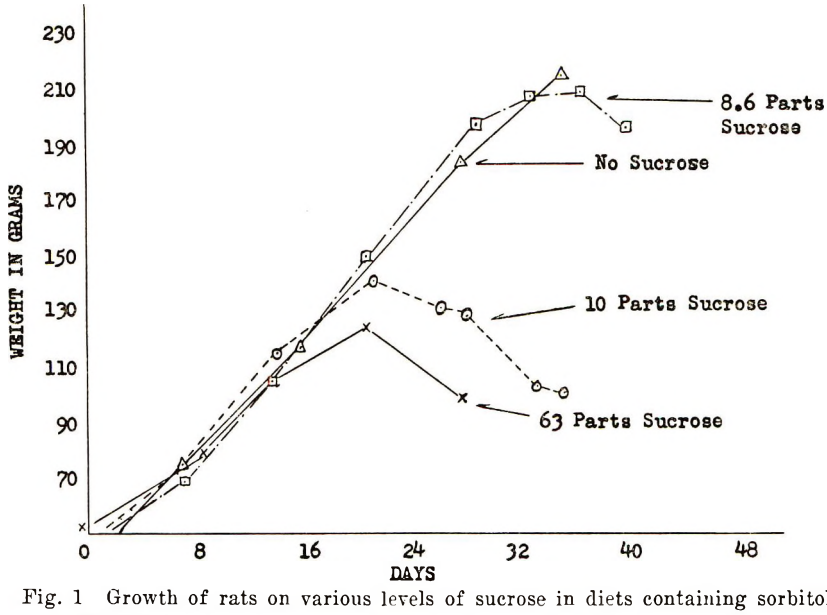

Fig. 1 Growth of rats on various levels of sucrose in diets containing sorbitol but no thiamine.

in the absence of sucrose. The maximum weights attained varied more or less inversely with the level of carbohydrate in the diet (fig. 1).

Sorbitol and antibiotics. In all of the thiamine-deficient diets studied, regardless of sucrose content, the stimulation of growth by 21 days due to penicillin was practically nullified when sorbitol was present in the diet (table 1, lines 2, 5, 6, 9, and 13). During this time-interval the growth stimulation due to sorbitol was reduced somewhat but was not eliminated by penicillin (table 2, lines 3, 4, 11, 12 and 16). This was true

even though maximum growth was not being realized. Data at 35 days, however, showed that penicillin and sorbitol were supplementary in their action, greater gains being realized when both supplements were present than when either was present in the diet alone (table 2, lines 3, 4, 11, 12 and 16).

Aureomycin, chloromycetin and streptomycin were inactive or had an adverse effect on sorbitol-free diets, but substantial increases in growth due to sorbitol occurred in the presence of these agents (table 2, lines 5–8). Bacitracin increased growth slightly at 21 days both in the presence or absence of sorbitol; at 35 days the increases due to bacitracin were substantial (table 2, lines 2 and 8).

DISCUSSION

It has long been recognized that the thiamine requirement depends upon the proportions of protein, fat, and carbohydrate in the diet. The present data confirm the older conclusions that fats have a sparing action on thiamine but do not completely eliminate the need for dietary thiamine, while in the presence of adequate dietary thiamine fats produced no better growth than carbohydrate. The thiamine-sparing action of fat could conceivably be due to two mechanisms, a decreased need for the vitamin (Banerji and Yudkin, '42) and a possible increased intestinal synthesis of the vitamin (Whipple and Church, '35). Much evidence favors the former mechanism.

Antibiotics (Guggenheim et al., '53; Monson et al., '54; Jones and Baumann, '55) and sorbitol (Yudkin and Morgan, '57) have been shown to increase the intestinal synthesis of various B vitamins, and this appears to be the explanation for the effects observed in the present study. It is unlikely that penicillin or sorbitol increased the availability of thiamine within the digestive tract, since the diet contained no thiamine, and hence a sparing of dietary thiamine, in the strict sense of the term, was impossible. The data suggest that sorbitol and penicillin act in a similar manner, and that they may even complement each other. The apparent effect of penicillin was

greater under less drastic conditions, namely, high-fat, low-carbohydrate diets, when the needs for thiamine were reduced. The fact that penicillin has not always appeared to be beneficial on completely thiamine-deficient diets in the past is probably due to the high carbohydrate content of the diets used.

Since the metabolism of sorbitol is via oxidation to fructose (Blakely, '51) and eventually to pyruvic acid, the protective effect of the sugar-alcohol must be other than to diminish the need for thiamine by the metabolizing tissues. Rather, an increased intake of sorbitol might actually be expected to increase the need for thiamine, compared to that with a diet high in fat or protein. The more significant characteristic appears to be the fact that sorbitol is absorbed slowly (Wick et al., '51) and thus remains in the intestine long enough to favor organisms that can use it. In the presence of sorbitol the entire digestive tract is increased in size and the contents are more fluid. The reported increase in the intestinal synthesis of thiamine (Yudkin and Morgan, '57) apparently depends upon this change, and the over-all effect persists even in the presence of penicillin (table 2).

In contrast to penicillin, the antibiotics aureomycin, chloromycetin and streptomycin did not stimulate growth when thiamine and sorbitol were absent from the diet, but aureomycin did stimulate growth when sorbitol was present. Apparently the organisms that develop in the presence of sorbitol are sensitive to aureomycin which presumably "spares" the newly synthesized vitamin but is not the cause of its synthesis.

SUMMARY

Penicillin, sorbitol, or both in combination were added to diets varying in carbohydrate and fat but lacking in thiamine, and the effectiveness of the additives was determined on the growth of rats. Either sorbitol or penicillin consistently increased the growth and survival of thiamine-deficient rats, the magnitude of the growth increase being greatest when the

need for thiamine was reduced, namely, on high-fat diets. After longer intervals on the diets there was a supplementary action of penicillin on the effectiveness of sorbitol.

The results confirm the report of Yudkin and Morgan ('57) in that rats fed sorbitol grow in the absence of thiamine; it appears that penicillin and sorbitol act in a similar manner, increasing the intestinal synthesis of thiamine.

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EFFECT OF BOUND GOSSYPOL ON THE GROWTH-PROMOTING PROPERTIES OF COTTONSEED, SOYBEAN AND PEANUT MEALS[1]

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Cottonseed meals having about equal amounts of free gossypol may effect marked differences in growth when fed to animals. Consequently, factors other than free gossypol *per se* apparently are involved in the growth response. Some of these differences in nutritional value have been attributed to variations in processing procedures (Olcott and Fontaine, '41; Sure et al., '53). Drastic processing treatment not only lowers the free-gossypol content of the meals but also reduces the availability of amino acids (Kuiken, '52; Sure et al., '53) and decreases nitrogen solubility (Lyman et al., '53).

During the processing of cottonseed, most of the free gossypol is converted to the bound form, presumably by condensation with the amino groups of the protein (Withers and Carruth, '18; Clark, '28). Bound gossypol is considered to be relatively inactive physiologically since the toxicity of well-cooked cottonseed meal is very low. Meal of this type adequately supplemented with powdered milk had little detrimental effect on rats (Withers and Carruth, '18). These workers believed that "D-gossypol," now known as bound

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gossypol, was injurious to rabbits and swine, but Sewell ('43) did not observe any ill effects from feeding well-cooked cottonseed meal to swine. Lyman et al. ('44) obtained good growth in guinea pigs fed cottonseed meal containing 0.024 to 0.052% free gossypol and 0.74 to 0.77% total gossypol. The weight of evidence to date, however, indicates the possibility that bound gossypol may be a factor affecting the growth response of animals fed cottonseed meal (Withers and Carruth, '18; Lyman et al., '53; Baliga and Lyman, '57).

EXPERIMENTAL

The study consisted of two parts: First, the development of a procedure for binding free gossypol to the oil-seed meals, and, second, the biological assay of the meals.

Part I. Binding the gossypol to the meals

The determination of the effect of bound gossypol on the nutritive value of cottonseed meal was dependent upon obtaining a meal with a low initial bound-gossypol content to be used in the control diets and to serve as a starting material to which gossypol could be bound to the extent of approximately 1.1% (air-dry basis), about the average for commercial meals in this geographical area.

In the binding process, measures were taken to minimize deleterious changes of the meals and to reduce the free gossypol to 0.05% or less.

Procedure. Cottonseed meal. Finely ground ether-extracted cottonseed flakes, subsequently referred to as cottonseed meal, containing 0.045% free and 0.293% bound gossypol were used as the raw material. To a solution of 13.76 gm of gossypol in 1500 ml of peroxide-free ether was added 1090 ml of 95% ethanol and 385 ml of water. (The solvent was approximately 46.3% ether, 37.2% ethanol and 16.3% water.) The gossypol to 0.05% or less.

mixing bowl of a mechanical mixer and mixed for 15 minutes at low speed at room temperature. A steam-heated water

bath at 60°C was placed under the bowl and the mixing continued for 2.75 hours. The meal was removed and air-dried at room temperature. Since some of the gossypol remained free, the meal was washed three times with 1500 ml of a mixture containing 90% ether and 10% alcohol by volume and filtered on a Buchner funnel under vacuum to remove the unbound gossypol. The gossypol content of the washed meal is shown under diet 2, table 1. The free gossypol was determined by the method of Smith ('46) and the bound as the aniline derivative by a spectrophotometric method developed by Smith ('58).

Soybean meal. Gossypol was bound to finely-ground solvent-extracted soybean meal by the method previously described under cottonseed meal. The amount of gossypol added in the solvent mixture was increased to 19.2 gm since gossypol does not occur in soybean meal. The free and bound gossypol content of this meal is shown under diet 5, table 1.

Peanut meal. Considerable difficulty was encountered in binding gossypol to peanut meal in preliminary studies. A batch of 1600 gm of finely ground solvent-extracted peanut meal was treated as previously described for the other meals, with 20.5 gm of gossypol. The resulting product contained 0.428% of free gossypol. Washing the product three times as described for cottonseed meal reduced the free gossypol to 0.295%, which was considerably higher than in the other meals. The gossypol content was as shown under diet 8, table 1.

A second batch of peanut meal plus gossypol was prepared as follows: To 500 gm of peanut meal in a 3-liter flask was added a mixture of 600 ml of ether, 756 ml of alcohol and 120 ml of water containing 7.25 gm of gossypol. The flask was put into a water bath and the contents were mixed constantly until most of the ether boiled off. Then a condenser was placed into the flask, and the mixture was heated for three hours at 65 to 73°C. The mixture was filtered on a Buchner funnel under vacuum, washed with 500 ml of hot alcohol while

suspended in a beaker and then filtered. The gossypol content of the dried residue was as shown under diet 10, table 1.

Batches of cottonseed, soybean and peanut meals, respectively, were given treatments identical with that used in binding the gossypol to the various meals, but without the addition of gossypol. These meals were used in control diets to determine the effect of the binding process. Batches of soybean

TABLE 1

Percentage of gossypol in the diets (Phase 1) based on analysis of the meals at the beginning of the experiment and of the diets at the close of the feeding experiments

DIET [1]		GOSSYPOL IN THE MEALS		CALCULATED GOSSYPOL IN THE DIET		GOSSYPOL BY ANALYSIS OF THE DIET	
Oil meal	No.	Free	Bound	Free	Bound	Free	Bound
		%	%	%	%	%	%
Cottonseed meal	1	0.045	0.293	0.010	0.063	0.015	0.051
	2	0.129	1.097	0.028	0.237	0.029	0.213
	3	0.045	0.293	0.010	0.066	0.012	0.057
Soybean meal	4	—	—	—	—	—	—
	5	0.091	1.013	0.019	0.206	0.021	0.183
	6	—	—	—	—	—	—
Peanut meals	7	—	—	—	—	—	—
	8	0.295	0.822	0.071	0.197	0.077	0.197
	9	—	—	—	—	—	—
	10	0.140	1.060	0.030	0.231	0.048	0.237
	11	—	—	—	—	—	—

[1] Diets Nos. 4, 6, 7, 9 and 11 were controls without added gossypol.

and of peanut meals were washed with the ether-alcohol mixture and used as untreated controls. The cottonseed meal was not so washed because it already had been exhaustively extracted with ether.

Discussion of preparation procedure. The gossypol solution was prepared by dissolving the gossypol in the proper amount of ether, then adding alcohol and finally water to give a homogenous mixture (Marquerol and Goutal, '17). Water was

included in the solvent mixture for binding gossypol to the meal since moisture appears to be essential in producing cottonseed meals of low free-gossypol content by commercial methods. Small amounts of water change the physical characteristics of the meal by softening the particles, which process presumably should stimulate the reaction between the meal and gossypol. The ethanol in the solvent mixture serves to make the water and ether miscible, to disperse the water uniformly through the meal while mixing and to act as a solvent for any gossypol not combined with the meal after the evaporation of the ether. The ether serves as a solvent for the gossypol while dispersing it uniformly over the meal by mixing in a mechanical mixer.

The ether gradually evaporated during the mixing at room temperature and rapidly after the hot-water bath was placed under the bowl. Most of the alcohol evaporated before the heating was discontinued. A large exhaust fan to insure good ventilation was used to prevent the accumulation of solvent vapors in the laboratory.

Heating appears to be essential for the combination of gossypol with the meal. This is substantiated by the fact that the gossypol in industrial processing in almost completely combined with the meats after cooking and before the oil is expressed by the hydraulic press. Laboratory observations indicated that residual free gossypol was lower in preparations in which the operation was carried out for 4 hours while the water bath was gradually raised from about 60° to 80° to 85°C and the meal to about 73° to 74°C.

Part II. Biological assay of prepared meals

This part consisted of two phases: The first involved the response of weanling rats to diets containing the various prepared meals, and the second, the response of rats to cottonseed meals with and without bound gossypol, supplemented with amino acids.

Phase 1. Comparison of effects from different meals

Phase 1 consisted of two trials. In the first, the free gossypol level in the control diet was not adjusted to the level in the diets containing the corresponding meals with bound gossypol. In the second, free gossypol was added to the control diets in amounts equivalent to that in diets containing bound gossypol.

Trial 1. Comparisons without free gossypol adjusted in control diets. This comparison was designed to show the effect of the binding treatment and of bound gossypol on the growth of rats. Consequently, each meal containing bound gossypol was compared with the same meals without any treatment and with the same meals treated for binding without gossypol, tables 1 and 2.

TABLE 2

Effect of bound and free gossypol in dietary proteins on the average weight gains of rats during a 4-week period (Phase I)

OILSEED MEAL	NO. OF RATS	TREATMENTS					
		None		For binding			
				With added gossypol		Without gossypol	
		Diet no.	Av. wt. gains	Diet no.	Av. wt. gains	Diet no.	Av. wt. gains
			gm		*gm*		*gm*
Cottonseed meal	10	1	63.1	2	37.0	3	57.3
Soybean meal	10	4	58.1	5	45.2	6	60.3
Peanut meal	10	7	23.7	8	5.1	9	18.7
	5			10	6.8	11	23.4

$L.S.D._{.05} = 9.49$.
$L.S.D._{.01} = 13.80$.

A. Diets. The diets contained 10% protein ($N \times 6.25$), which was supplied by the respective meals; 5% fat supplied by the fat in the ingredients plus vegetable shortening,[2] 3% Wesson salt mixture; 0.5% cod liver oil and sufficient starch to bring the ingredients to 100%. Each kilogram of diet was supplemented with 1.5 mg of thiamine hydrochloride, ribo-

[2] Crisco.

flavin, pridoxine hydrochloride and calcium pantothenate, respectively, and 500 mg of choline chloride. The reserve portions of the diets were stored in the refrigerator.

The amount of free and of bound gossypol found in the diets 8 weeks after preparation was in close agreement with the amount added, as calculated from the analysis of the meals, table 1. From these data the free and the bound gossypol apparently were stable in the diets.

B. Design of test. The meals listed in table 1 were assayed in comparative feeding trials in which the diets containing the respective meals were fed ad libitum to weanling rats during a 4-week period. Each of the diets 1 through 9, table 2, was fed respectively to 5 rats grouped in a randomized block design. This series was repeated, and diets 10 and 11, table 2, were added to determine the effect of lower free gossypol and higher bound gossypol in peanut meal than the corresponding amounts in the meal used in diet 8, table 2. All peanut meals were supplemented with 0.1% of DL-methionine after the first week in an effort to overcome the poor growth of the rats receiving this meal.

C. Results and discussion. Results from feeding the various oil-seed meals are shown in table 2. Statistical analysis of the data showed that the binding treatment *per se* had no significant effect on growth. If the low level of free gossypol in the diets could be discounted, comparative results indicate that bound gossypol in each of the three meals effected a highly significant depression in growth in all comparisons except in diet 4 vs. diet 5, in which the difference was signified at $P < 0.05$. There were no differences between responses from cottonseed meal and soybean meal. While the depression of growth from gossypol-treated peanut meal was similar to that from gossypol-treated cottonseed or soybean meal, the growth of the rats receiving peanut meal was markedly lower than that from the other meals (diets 8 and 10 vs. 2 and 5, table 2). The lower gains from the peanut meal control diets compared with those from corresponding control diets of

either cottonseed or soybean meal were highly significant, $P < 0.01$, (diets 7, 9 and 11 vs. 1 and 3, and 4 and 6, table 2).

Based on the findings of Carruth ('47), Ingram et al. ('50), Milligan and Bird ('51), Kuiken ('52) and Sure et al. ('53), the poor growth from gossypol-treated meals may be due to lowered availability of certain amino acids. It has been postulated that gossypol combines with amino acids of protein in much the same way that it combines with aniline (Withers and Carruth, '18; Clark, '28; Carruth, '47). Carruth ('47) also found that the protein-gossypol combination is somewhat resistant to digestion.

Since some of the amino acids are quite reactive, especially lysine and to some extent methionine and tryptophan, they possibly might combine with gossypol in the same manner as other amines. Such a reaction may reduce the availability of the amino acids and thus lower the growth response of rats fed meals containing bound gossypol.

The poor growth response from peanut meal indicates that the quality of the protein in this meal is inferior to the protein in cottonseed and in soybean meals. However, supplementation of the peanut-meal diets with 0.1% of DL-methionine effected no distinct improvement in growth rate.

Diet 8, containing peanut meal to which gossypol was bound, possibly contained sufficient free gossypol to affect the growth rate adversely when the dietary protein level is as low as 10% (Andrews, '48). Consequently, a second batch of gossypol treated peanut meal was prepared, having a lower free gossypol content than did the meal in the first batch (diet 10, table 1). A comparison of this meal in diet 10 (table 2) with a meal similarly treated without added gossypol, diet 11, again revealed a marked suppression of growth by the gossypol-treated meal (table 2). Furthermore, comparisons of growth of rats fed gossypol-treated peanut meal in a diet containing 0.071% of free gossypol, diet 8, with a similar meal in a diet containing 0.030% of free gossypol (diet 10, table 2) showed only a slight and insignificant difference. Therefore, it was concluded that the higher level of free gossypol in diet 8 had

little or no effect on the growth of the rats. Low food intake may have obscured any effects from free gossypol.

The foregoing results indicate that bound gossypol as a part of either cottonseed, soybean or peanut meals and present in diets in concentrations ranging from 0.183 to 0.237% lowered the growth response of rats significantly when the dietary level of protein was only 10%. This cannot be considered conclusive, since the free gossypol associated with the bound was not counterbalanced in the control diets.

Trial 2. Effect of bound gossypol in cottonseed and soybean meals with the control and test diets equivalent in free gossypol. To ascertain whether or not the small amount of free gossypol associated with the bound gossypol might have suppressed growth, further testing was conducted.

A. Preparation of meals. Additional batches of cottonseed and soybean meals were prepared with and without added gossypol as previously described except for the following: the residual oil was extracted from the soybean meal with ether. For 1000 gm of meal, a mixture of 1600 ml of ether, 715 ml of ethanol and 240 ml of water was used. The binding operation was carried out for 4 hours while the temperature of the water bath was gradually raised from about 60° to 80 to 85°C. The final temperature of the meals was 73 to 74°C. Moreover, the treated meals were not washed with the alcohol-ether mixture. The gossypol contents of the cottonseed and the soybean meals are shown in table 3. Nitrogen solubility for the meals, determined by the method of Lyman et al. ('53), is shown in table 3.

B. Feeding the meals. The meals listed in table 3 were incorporated into diets 24 to 27 and 28 to 31. The composition of these diets was identical with that of the diets used in trial 1, except a refined cottonseed oil [3] was substituted for Crisco, and each kilogram of diet was supplemented with the following vitamins in milligrams: thiamine hydrochloride 5, riboflavin 5, pyridoxine hydrochloride 5, calcium pantothenate 20, niacin 30, *p*-aminobenzoic acid 30, and choline chloride

[3] Wesson.

TABLE 3

Effect of bound gossypol on weanling rats receiving the control and the test diets equivalent in free gossypol

OIL MEALS			DIET NO.	N SOLUBILITY OF MEAL	RATS/DIET	GOSSYPOL IN				AV. WEIGHT GAIN FOR RATS
Kind	Treatment no.	Treatment				Meal		Diet		
						Free	Bound	Free	Bound	
				%		%	%	%	%	*gm*
Cottonseed	1	None	24	85.8	8	0.092	0.224	0.0169	0.0411	83.5
	2	Treated for binding, no added gossypol	25	85.2	8	0.019	0.321	0.0198	0.0579	81.5
	3	Treat No. 2 + free gossypol = to that in diet 27	26	85.2	8	0.019	0.321	0.0067	0.0579	84.3
	4	For binding with added gossypol	27	58.5	8	0.037	1.143	0.0067	0.2078	65.3
Soybean	1	None	28	94.0	8	—	—	—	—	76.5
	2	For binding, no added gossypol	29	80.7	8	—	—	—	—	73.5
	3	Treat No. 2 + free gossypol = to that in diet 31	30	80.7	8	—	—	0.0169	—	76.3
	4	For binding with added gossypol	31	61.1	8	0.086	1.159	0.0165	0.2223	64.4

$L.S.D._{.05} = 10.1$.

$L.S.D._{.01} = 13.5$.

1000. Sufficient pure gossypol was added to the non-gossypol-treated meals to equal the residual free gossypol in the gossypol-treated meals. The gossypol was dissolved in ether, mixed with the Wesson Oil and then blended with the other ingredients, by means of a mechanical mixer, until the ether had evaporated and the diets were thoroughly mixed.

These diets were individually fed ad libitum to 8 replications of weanling rats according to a randomized-block design.

C. Results and discussion. The results presented in table 3 show that for cottonseed meals and soybean meals, respectively, there were no differences among the meals that did not contain bound gossypol. The gains from the diets containing the bound gossypol were significantly lower than from the control diets, $p < 0.01$ for cottonseed meal and $p < 0.05$ for soybean meal. Thus, the results of trial 2 indicate the correctness of the assumption that the free gossypol in the diets of trial 1 had little if any effect on growth. The observations from both of these trials (phase 1 of this study) are in agreement with the findings of Baliga and Lyman ('57) who reported that bound gossypol lowers the nutritive value of cottonseed meal. The reduction in nitrogen solubility associated with the gossypol binding (table 3) is also in agreement with the observations of Baliga and Lyman ('57).

Phase II. The effect of amino acid on the growth-promoting properties of cottonseed meal containing bound gossypol.

Supplementation with the appropriate amino acid or acids should improve the growth response from cottonseed meals containing bound gossypol, if the gossypol is bound to the protein and thus reducing the availability of these nutrients. Cottonseed meal diets supplemented with amino acids were fed to elucidate this hypothesis.

A. Procedure. In this study the amino acids, lysine, methionine and tryptophan, either individually, or in combination, were incorporated into diets prepared from cottonseed meal treated for binding either with or without added gossypol. The diets containing 10% protein were similar to those fed in

phase 1, trial 1, except amino acid additions replaced an equal amount of starch (table 4). In addition, the cottonseed meal treated for binding, with and without added gossypol, was compared at the 20% protein level. In design, this test was the same as described in phase 1, trial 1.

B. Results and discussions. The average weight gain of the rats in phase 2 are shown in table 5. The growth, at the 10% protein level, from cottonseed meal containing bound gossypol (diet 12) was significantly lower ($P < 0.01$) than

TABLE 4

Supplementation of the cottonseed meal diets with amino acids (Phase 2)

PROTEIN LEVEL	DIET NO.	COTTONSEED MEAL IN DIET	TOTAL GOSSYPOL IN DIET	AMINO ACID ADDED		
				Methionine	Lysine	Tryptophan
%		%	%		% of diet	
10	12	18.52	0.207	0	0	0
	13	18.52	0.207	0.4	0	0
	14	18.52	0.207	0	0.5	0
	15	18.52	0.207	0	0	0.3
	16	18.52	0.207	0.4	0.5	0.3
	17	18.63	0.068	0	0	0
	18	18.63	0.068	0.4	0	0
	19	18.63	0.068	0	0.5	0
	20	18.63	0.068	0	0	0.3
	21	18.63	0.068	0.4	0.5	0.3
20	22	37.04	0.414	0	0	0
	23	37.25	0.136	0	0	0

that treated for binding without added gossypol (diet 17). This is in agreement with results from phase 1 of this study.

The addition of methionine (diets 13 and 18), tryptophan (diets 15 and 20), or lysine, methionine and tryptophan in combination (diets 16 and 21) did not improve the growth significantly, as indicated by comparison of the foregoing diets with diets 12 and 17 (table 5). Either the bound gossypol did not affect the availability of methionine and tryptophan or sufficient amounts remained to meet the requirements for growth. Kuiken ('52), however, showed that heating cottonseed with Wesson oil and gossypol lowered the availability of

lysine, methionine and other amino acids, and Sure et al. ('53) observed an increased growth response when cottonseed meal diets were supplemented with lysine or methionine alone or in combination.

When lysine alone was fed with the meal containing bound gossypol (diet 14), the increase in growth was highly significant when compared with the same meal without lysine

TABLE 5

Effect of bound gossypol in cottonseed meal, with or without amino acid supplementation, on the average weight gains of rats during a 4-week period (Phase 2) [1]

LEVEL OF PROTEIN IN DIET	AMINO ACIDS ADDED	WEIGHT GAINS FROM DIFFERENT DIETS					
		With added gossypol			Without added gossypol		
		Diet no.	No. of rats	Gains	Diet no.	No. of rats	Gains
%				*gm*			*gm*
10	None	12	7	47.14	17	8	61.43
	Methionine, 0.4%	13	5	45.00	18	5	63.40
	Lysine, 0.5%	14	5	55.00	19	5	63.00
	Tryptophan, 0.3%	15	5	49.20	20	5	59.20
	Methionine, 0.4% Lysine, 0.5% Tryptophan, 0.3%	16	5	46.50	21	7	63.00
20	None	22	7	74.00	23	7	78.00

$L.S.D._{.05} = 5.34$.
$L.S.D._{.01} = 7.11$.

[1] All the diets in this phase of the study were subjected to the treatment used for binding gossypol.

(diet 12). Since lysine did not affect the growth response of rats fed the cottonseed meal treated for binding (without the addition of gossypol) but gave a marked improvement when added to the same meal to which gossypol was bound, it appears that binding gossypol to the protein lowered the availability of lysine. Other investigators (Lyman et al., '53; Sure et al., '53; Kratzer et al., '55; Miner et al., '55) also observed improved growth from lysine supplementation; however, the

heat treatment of the meals used by these investigators might have contributed to the effects noted (Olcott and Fontaine, '41). Observations that binding gossypol to cottonseed meal by heating did not reduce the amino acid availability appreciably unless oil was present (Kuiken, '52) do not seem to be in accord with the results in this study. Furthermore, the binding treatment *per se* apparently did not affect the nutritive quality of the meals (diets 1 vs. 3, table 2; diets 24 vs. 25 and 26, table 3).

The availability of either amino acids other than those tested or some other nutrient might have been affected by gossypol binding; otherwise the growth response from lysine supplementation should have been the same as for the treated meal without gossypol (diets 14 vs. 17, table 5). Another possibility is that lysine was not added at the optimum level. The lack of response to lysine when fed with methionine and tryptophan (diets 12 vs. 16) might have been due either to an amino acid imbalance or to a difference in the metabolism of lysine when these three amino acids were present in a free state. Pfander and Tribble ('55) found that supplementing with either lysine, methionine or tryptophan alone improved growth rates and feed efficiency of swine, but the three in combination were not so effective as lysine alone. These investigators suggest that the free amino acids in the diet may be absorbed and metabolized prior to the hydrolysis and absorption of amino acids in the feed protein, and possibly would not be available for synthesis into animal protein.

The exact mechanism by which gossypol is bound to the protein of cottonseed meal is not known. The combination of gossypol with the free amino groups of the amino acids to form rather stable compounds analogous to dianilino gossypol seems to be the most logical theory (Withers and Carruth, '18; Clark, '28; Carruth, '47). These stable compounds may pass unaffected through the digestive tract thus carrying the bound amino acids along with the gossypol.

The growth of rats fed cottonseed meal containing bound gossypol was 57% higher at the 20% protein level (diet 22)

than at the 10% level (diet 12). At the 20% level of protein in the diet, the difference in response to cottonseed meal with bound gossypol (diet 22) and without (diet 23) was not significant at the $P = 0.05$ level. At the 10% protein level, the treated cottonseed meal without bound gossypol (diet 17) produced less growth than the same meal with bound gossypol fed at the 20% protein level (diet 22). Probably cottonseed meal at this higher protein level supplied nearly adequate amounts of amino acids in spite of the bound gossypol.

The implications from this study are that the removal of the gossypol, if this can be done economically without a deleterious effect on the meal, is preferable to converting it to bound gossypol. Other properties being equal, the ideal cottonseed meal is low in both free and bound gossypol.

SUMMARY

The objective of this study was to determine the effect of gossypol bound to oil-seed meals on the growth-promoting properties of the meals.

A method for binding the gossypol to the meal without impairing the apparent growth-promoting properties of the meal in the absence of gossypol has been developed.

Cottonseed, soybean and peanut meals, untreated and treated for binding either with or without added gossypol, were compared in diets in which the respective meals supplied all the protein at the 10% level.

The meals were assayed by feeding the various diets to weanling rats during a 4-week period.

There were no differences in the growth of rats consuming the untreated and treated control meals. The growth from the meals supplying the bound gossypol at the rate of 0.183 to 0.237% of the diet was significantly lower than growth from the corresponding controls.

Cottonseed meals, treated for binding with or without added gossypol, were supplemented with either lysine, methionine or tryptophan individually or the three in combination. Improve-

ment of growth was reflected significantly only from lysine supplementation of the diets containing bound gossypol.

At the 20%-protein level in the diet, there was little difference in the growth response from cottonseed meal with or without bound gossypol, indicating that at higher protein levels the amino acid requirements are more adequately met than at the 10% protein level.

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DIETARY STUDIES ON SALIVARY PROTEASE ACTIVITY IN CARIES-SUSCEPTIBLE RATS [1]

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Stimulated saliva of mature Hunt-Hoppert caries-susceptible rats has been shown by Willett et al. ('57) at Michigan State University to possess approximately three times as much protease activity as that of the Hunt-Hoppert resistant animals. This finding was confirmed in the Harvard laboratories with representatives of the Hunt-Hoppert caries-susceptible and caries-resistant strains (Willett, Resnick and Shaw, '58). However, in the case of rats from the caries-susceptible and caries-resistant strains that had been independently developed at the Harvard School of Dental Medicine, a random distribution in the values for salivary protease activity was observed to occur without respect to either the caries-susceptibility of the strain or to the sex of the subjects (Willett, Resnick and Shaw, '58). These observations suggested the likelihood that the association of salivary protease activity with caries-susceptibility in the Hunt-Hoppert rats had occurred as the result of a coincidental genetic change that had not taken place in the Harvard animals.

The purpose of the present investigation was to provide more definitive information concerning possible relationships

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between salivary protease activity and caries-susceptibility in the case of the Harvard and Hunt-Hoppert caries-susceptible strains. In particular, information was needed to determine whether the presence or absence of carious lesions in the subjects at the times when salivary samples were collected influenced the levels of salivary protease. These experiments were carried out in both the Hunt-Hoppert and Harvard caries-susceptible strains through comparisons of the influence of a highly cariogenic diet with the influences of two carbohydrate-free, non-cariogenic diets to determine if any alterations in the protease activity of the saliva were caused by the diets directly or by the prevention of carious lesions.

PROCEDURE

The rats used in the first two experiments were representative offspring of the Harvard caries-susceptible strain. Forty-five rats were used in the first experiment and 60 rats in the second experiment. Forty-four rats of the Hunt-Hoppert caries-susceptible strain were used in the third experiment. The design in the three experiments was identical, with careful distribution of the rats into three comparable groups with respect to sex, weight and litters. The composition of the diets is presented in table 1. The rats in the first group of each experiment were fed our usual caries-producing diet, 700 + 15% of Cellu flour. The rats in the second and third groups were maintained on diets 770 + 15% of Cellu flour [3] and 751 + 15% of Cellu flour, respectively. These diets are identical with diet 700 + 15% of Cellu flour except that 27 and 36% of sucrose, respectively, have been replaced isocalorically by lard, and the remainder of the sucrose replaced in each case by the same weight of casein. Carbohydrate-free diets such as 770 + 15% of Cellu flour have previously been shown to be incapable of causing the initiation and progression of carious lesions (Shaw, '54).

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

Whole saliva was collected by the method of Benarde et al. ('56) after anesthesia [4] and pilocarpine stimulation. Prior to injection with pilocarpine, the teeth had been vigorously brushed with a small brush dipped in distilled water to reduce contamination by fecal matter, sawdust, and retained food particles. Salivary protein and protease determinations were carried out on individual whole saliva samples at each of three age levels, 60 to 65, 100 to 110, and 190 to 200 days of age. Salivary protease and salivary protein determinations

TABLE 1

Composition of diets

INGREDIENT	700 + 15% CELLU FLOUR	770 + 15% CELLU FLOUR	751 + 15% CELLU FLOUR
	gm	*gm*	*gm*
Sucrose	670	—	—
Casein (with added vitamin B-complex) [1]	240	240	240
Casein	—	400	310
Lard	—	120	160
Salt mixture [1]	40	40	40
Corn oil (with added fat-soluble vitamins) [1]	50	50	50
Desiccated liver	40	40	40
Cellu flour [2]	150	150	150

[1] J. Dent. Res., *27:* 47, 1948.

[2] Chicago Dietetic Supply House, Chicago, Illinois.

were made by the procedures described by Willett, Resnick and Shaw ('58). At the conclusion of each experiment, the animals were sacrificed and the caries score of each animal evaluated according to the method of Shaw et al. ('44).

In view of the variability in salivary protease values within any control or experimental group in these experiments, a 4th experiment was undertaken to determine the extent of this variation in individual rats if samples of saliva were collected repeatedly and to determine if this variability could be reduced by any alteration in the conditions imposed at the time of saliva collection. For this purpose a group of 31 rats of

[4] Nembutal, Abbott, was used in all cases where the rats were anesthetized.

the Harvard caries-susceptible strain was maintained from weaning on ration 700 + 15% of Cellu flour. Beginning on the 60th day of age, saliva was collected 10 times at approximately weekly intervals and used for salivary protease determinations. The first three collections on the 60th, 67th and 74th days of age were made under the usual conditions followed in the first three experiments, namely, anesthesia, pilocarpine stimulation, no withdrawal of food prior to saliva collection, and a cleaning of the teeth with a small brush and distilled water. After the third collection, the rats were divided into two groups, A and B, with an equitable distribution according to littermates, sex and previous protease values. The rats in group A remained as controls with saliva collections in the usual fashion. The collection of saliva from the rats in group B on the 81st, 88th and 95th days of age was made after 16 hours of starvation, without any cleaning but under anesthesia with pilocarpine stimulation. The collection of saliva from the rats in group B on the 109th, 123rd and 130th days of age was made after 16 hours of starvation, with the usual cleaning process, and under anesthesia with pilocarpine stimulation. Saliva was collected from the control rats on the same days as the experimentals throughout. The 10th collection from control and experimental rats was made on the 137th day of age under the usual collection procedures of cleaning without a preparatory food withdrawal period.

In addition to the above experiments, preliminary studies have been undertaken to determine whether rats with unusually high salivary protease values could be selected from the Harvard caries-susceptible strain for the development of a substrain that would have a high caries-susceptibility as well as a high salivary protease activity. Simultaneously rats with unusually low salivary protease values were selected from the Harvard caries-susceptible strain for an attempt to develop a second substrain that would have an equally high caries-susceptibility simultaneously with a low salivary protease activity. Most of the rats for these breeding trials were taken from the general colony. However, the two rats with the

lowest preliminary salivary protease values were removed from the 4th experiment after the 6th experimental period. The two rats with the highest preliminary salivary protease values were removed from the experiment after the 7th experimental period. These rats were added to the respective groups being bred for the development of a low salivary protease substrain and of a high salivary protease substrain.

RESULTS

The rats in each of the three groups in the three dietary experiments grew and developed normally and had reached mature adult body weights prior to the termination of the experimental period. The extent of carious lesions is presented in table 2. The incidence of dental caries was very high among the rats in group 1 of each experiment which had been maintained on ration 700 + 15% of Cellu flour. As would be expected from previous experiments in this laboratory, the incidence of dental caries was appreciably higher among the Harvard caries-susceptible rats in groups 1 of the first and second experiments than among the Hunt-Hoppert caries-susceptible rats in group 1 of the third experiment. In striking contrast, all rats in groups 2 and 3 of the 3 experiments where carbohydrate-free diets, 770 + 15% of Cellu flour and 751 + 15% of Cellu flour, had been used were caries-free.

The values for salivary protease activity for the first three experiments are presented in table 2. The salivary protease activity values for the rats that received cariogenic diet, 700 + 15% of Cellu flour, did not differ significantly from the values for rats that received the non-cariogenic diets, 770 + 15% of Cellu flour and 751 + 15% of Cellu flour. This finding was true not only in the Harvard rats where no association between caries-susceptibility and salivary protease activity was observed (Willett, Resnick and Shaw, '58) but also in the Hunt-Hoppert rats where a strong association between caries-susceptibility and salivary protease activity has been reported (Willett et al., '57; Willett, Resnick and Shaw,

'58). The complete prevention of dental caries in both strains did not alter the level of salivary protease activity that was typical of each strain.

As observed in earlier studies, there was a high variability in the salivary protease values for any group of rats at different time intervals. In general among the Harvard caries-susceptible rats, the protease activity in the saliva increased with age to reach the maximum levels in the last period at which tests were made. This trend is much stronger among the

TABLE 2

Relation of diet to salivary protease activity and to dental caries scores in caries-susceptible rats [1]

DIET [2]	60–65 DAYS	100–110 DAYS	190–200 DAYS	EXTENT OF CARIOUS LESIONS
	Experiment 1 — Harvard susceptible strain			
700	109 ± 18 [3]	187 ± 44	151 ± 12	79.0 ± 7.5
	(15)	(15)	(14)	(14)
770	138 ± 26	220 ± 27	260 ± 38	0
	(16)	(16)	(13)	(13)
751	148 ± 38	191 ± 22	209 ± 33	0
	(14)	(13)	(8)	(8)
	Experiment 2 — Harvard susceptible strain			
700	132 ± 14	147 ± 13	182 ± 14	82.5 ± 8.0
	(20)	(20)	(17)	(17)
770	155 ± 15	153 ± 18	180 ± 15	0
	(18)	(14)	(13)	(13)
751	186 ± 19	224 ± 34	179 ± 15	0
	(22)	(18)	(15)	(15)
	Experiment 3 — Hunt-Hoppert susceptible strain			
700	163 ± 16	301 ± 43	339 ± 31	61.4 ± 7.5
	(14)	(16)	(12)	(12)
770	188 ± 19	196 ± 18	381 ± 63	0
	(13)	(12)	(11)	(11)
751	161 ± 21	254 ± 29	326 ± 32	0
	(15)	(12)	(11)	(11)

[1] All values expressed in terms of micrograms of tyrosine and its equivalents liberated during incubation. The number of animals in each age and diet group is within parentheses.

[2] Each diet was supplemented with 15% of Cellu flour.

[3] Standard error of the mean.

Hunt-Hoppert caries-susceptible rats where the salivary protease values are strikingly higher at 190 to 200 days of age than in the 60-65 day interval. The values among the Hunt-Hoppert rats in the 190 to 200 day period were much higher than for the Harvard rats of the same age. Again this difference between the Hunt-Hoppert and Harvard strains was inversely related to the susceptibility to carious lesions.

Protein determinations on the saliva of the rats in these experiments did not provide important information. A great deal of variation was noted between the rats within any group and between groups. Although the average salivary protein concentration for the various groups varied from 5.2 to 10.2 mg/ml, no significant correlation between diet and protein level, animal age and protein level, and genetic strain and protein level was observed.

The results of the 4th experiment are presented in table 3. The average salivary protease values for the first three collection periods, which were considered as preliminary to the experiment proper, were practically identical, 126, 130 and 130 μg, respectively. This close similarity of the average values at the three weekly intervals was gratifying and yet at the same time somewhat surprising. The wide range of salivary protease values encompassed in each average and the wide fluctuation for the three consecutive values for any individual rat would lead the investigator to anticipate difficulties in obtaining reproducible data. Yet with groups of this size, reasonable reproducibility from collection to collection had been obtained.

In previous studies on the variations in salivary protease values, conclusive increases with age have been reported in caries-susceptible strains (Willett et al., '57, '58). These increases were demonstrable in three dietary experiments and are again evident in this experiment for the collection periods from the 95th to the 137th days of age.

From an inspection of the data on the basis of consecutive values for individual rats, it was clear that an average of the three salivary protease values obtained for each rat in these

TABLE 3

Relation of conditions at time of saliva collection to salivary protease activity in caries-susceptible rats [1]

EXPERIMENTAL PERIOD AND GROUP	FOOD WITHDRAWAL	MOUTH CLEANED	NUMBER OF RATS	AVERAGE AGE	AVERAGE CURRENT SALIVARY PROTEASE VALUE	RANGE IN CURRENT SALIVARY PROTEASE VALUES	AVERAGE PRELIMINARY SALIVARY PROTEASE VALUES [2]	DIFFERENCE BETWEEN AVERAGE CURRENT AND AVERAGE PRELIMINARY SALIVARY PROTEASE VALUES [3]
				days				
1	No	Yes	31	60	126 ± 9 [4]	47–299	—	—
2	No	Yes	30	67	130 ± 12	44–302	—	—
3	No	Yes	30	74	130 ± 8	42–219	—	—
4A	No	Yes	12	81	129 ± 14	49–214	126 ± 9	3 (0.2)
4B	Yes	No	14	81	196 ± 26	92–512	135 ± 10	61 (2.2)
5A	No	Yes	13	88	140 ± 11	60–182	124 ± 9	16 (1.1)
5B	Yes	No	13	88	150 ± 13	65–248	139 ± 10	11 (0.7)
6A	No	Yes	11	95	177 ± 16	112–263	118 ± 9	59 (3.3)
6B	Yes	No	10	95	229 ± 18	140–344	144 ± 11	85 (4.0)
7A	No	Yes	9	109	248 ± 24	126–386	119 ± 9	129 (5.0)
7B	Yes	Yes	8	109	230 ± 36	120–471	136 ± 12	94 (2.5)
8A	No	Yes	8	123	193 ± 16	96–244	113 ± 8	80 (4.4)
8B	Yes	Yes	7	123	180 ± 16	110–226	127 ± 10	53 (2.8)
9A	No	Yes	8	130	211 ± 11	156–268	113 ± 8	98 (7.0)
9B	Yes	Yes	6	130	196 ± 18	130–246	129 ± 11	67 (3.2)
10A	No	Yes	8	137	224 ± 23	126–318	113 ± 8	111 (4.6)
10B	No	Yes	6	137	246 ± 36	106–368	129 ± 11	117 (3.1)

[1] All values expressed in terms of micrograms of tyrosine and its equivalents liberated during incubation.

[2] Preliminary salivary protease values were determined for each rat as an average of the values obtained for that rat in the first three collection periods. The average preliminary salivary protease values for a given group include values from only those rats for whom a salivary sample was collected in that experimental period. Thus this varies somewhat from group to group due to the different numbers of rats represented by reason either of loss of rats from the group or the inability to collect an adequate saliva sample in a given experimental period.

[3] Within parentheses is presented the critical ratio for each difference. By definition, the critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. When the critical ratio is less than 2.0, the difference between the means is judged to be statistically insignificant; when this ratio is between 2.0 and 2.9, the difference is of borderline significance; when the critical ratio is 3.0 or higher, the difference is considered to be highly significant.

[4] Standard error of the mean.

introductory evaluation periods was a much more accurate indicator of the range of salivary protease activity over that animal's lifetime than most single salivary determinations, Hence a preliminary salivary protease value was determined for each rat on the basis of the average of these three preliminary values. This preliminary salivary protease value was used to sort the available rats into groups A and B for the remainder of the experiment.

In comparison of all averages for the collection periods from 4 to 10, the average preliminary salivary protease values of the actual rats for which saliva was collected should be used. The latter averages fluctuated somewhat because of inability to obtain saliva from the occasional rat during the collection period, because of removal of 4 rats for breeding purposes, or because of deaths during or after the collection period, primarily due to problems in anesthesia or post-anesthesia pneumonia.

In the 4th, 5th and 6th collection periods, the customary procedure of feeding the rats up until the time of collection and then brushing the teeth carefully was followed in control group A; in comparison, a preliminary withdrawal of the food cups for 16 hours without any cleaning of the teeth was followed for the rats in experimental group B. In the experimental group at the 4th collection period, the increase in average salivary protease value from the preliminary periods to the current period appeared to be strikingly higher than for the control animals. Indeed, the average current salivary protease value for this group was unusually high for rats of this age and was accompanied by an unusually high variability. This high average resulted primarily from the absence of any rats with unusually low values and presence of one with an unusually high value (512 μg). The cause of this unusual distribution is not known. However, the average increases in the 5th and 6th periods were closely similar for the control and experimental groups. On the basis of the data from the 4th, 5th and 6th collection periods, there appears to

be no justification for believing that starvation for 16 hours without cleaning improved the saliva collection process.

In collection periods 7, 8 and 9, the rats in experimental group B had their food dishes removed 16 hours prior to saliva collection and their teeth brushed before the pilocarpine stimulation. In all three collection periods, the increase in salivary protease value from the preliminary periods to the current periods was consistently greater for the control rats than for the experimental groups. Although this difference was consistent, it was not statistically significant in any one of the three comparisons and did not appear to be associated with a reduction in the variability of the results within any average. Unusually high averages were obtained for both control and experimental groups at the 7th collection period. No complete explanation is available for these high values. However, to some extent the average for the control group during this collection period has been increased by reason of the previous withdrawal of two of the rats with the lowest salivary protease values for breeding purposes. Immediately after this collection period, the rat with the highest salivary protease value in each of group A and B was removed for the same purpose. However, in addition to these arithmetical influences upon the averages, presumably some unknown variable exists to influence salivary protease values which is beyond our present recognition and control. Since this procedure of cleaning the teeth after a period of food withdrawal has had no major influence upon the results, it could be used as an alternative to the customary procedure of cleaning without food withdrawal. However, no major case can be made to indicate that the former procedure is appreciably superior to the customary procedure.

In the 10th experimental period, the usual collection procedure was followed. The increase from the average preliminary to the average current salivary protease value is practically identical for the control and experimental groups.

In each one of the 7 possible comparisons between the control and experimental groups of rats in experiment 4 from the

4th to the 10th collection period, the difference in the average salivary protease levels between the experimental and control rats was not statistically significant except at the 4th collection period on the 81st day. Even in this exception, the difference was barely of borderline significance by reason of the very large standard error of the mean for the experimental group.

In corroboration of the evidence in the first three experiments of significantly increased salivary protease values with age, it is noteworthy that the increases in average salivary protease value from the preliminary to each current collection period were statistically significant beginning with the 6th collection period on the 95th day of age for both the control and experimental groups and continuing through the 10th collection period on the 137th day of age.

These data from the 4th experiment, while not minimizing the problems of variability within the salivary protease values, suggest that the consideration of series of averages of values from fairly large groups and the reference of these values to the initial salivary protease values of the same animals can lead to dependable results. However, confidence in any single determination or in some cases, in any single average without reference to others in the series may lead to erroneous impressions.

In the attempts to breed substrains with low salivary protease and with high salivary protease values, a substantial number of offspring have been obtained for the second generation after the original stock were selected for their desirable salivary protease values. On the basis of data presently available for the offspring, there is no clear suggestion that progress has been made toward the establishment of either a low salivary protease substrain or a high salivary protease substrain.

DISCUSSION

A sharp contrast exists between the observed association of salivary protease activity and dental caries susceptibility in

the Hunt-Hoppert strains of rats on one hand and the lack of association in these parameters among the representatives of the Harvard strains of rats on the other hand. This contrast led Willett, Resnick and Shaw ('58) to conclude that the tendency toward high salivary protease levels in the caries-susceptible Hunt-Hoppert rats appeared to be a genetically determined trait that has appeared simultaneously with the tendency toward high caries-susceptibility. On the same basis they concluded that salivary protease activity, *per se,* was not likely to be directly related to dental caries. The need for additional data of more conclusive nature led to the design of the present series of experiments in which major changes in the ratio of fat, protein and carbohydrate were made to test the influence upon salivary protease activity and in which carious lesions were prevented deliberately. The prevention of carious lesions was achieved by the use of carbohydrate-free diets as described by Shaw ('54), to determine whether open carious lesions and their flora had an influence upon the protease activity of the saliva.

Any major alteration in the salivary protease activity in those animals maintained on the non-cariogenic diets would theoretically imply some type of causal relationship between either caries-susceptibility and salivary protease activity or between the presence of carious lesions and the amount of salivary protease. Reduction in salivary protease activity might arise through decrease of certain microorganisms associated with caries which would ordinarily thrive in the carious lesion, or from other decaying organic materials present in the open lesion. If, on the other hand, the protease activity remained constant or increased with age even when carious lesions were completely prevented, independence of the two traits would be suggested. The fact that salivary protease activity in representatives of both the Harvard and Hunt-Hoppert caries-susceptible strains was comparable on the three diets at any given age in a single strain supports our conclusions from earlier data that the association between caries-susceptibility and salivary protease activity in the

Hunt-Hoppert strains is a coincidental genetic occurrence and that salivary protease activity is not directly related to dental caries.

The increase in salivary protease activity with age in both caries-susceptible strains and especially in the Hunt-Hoppert strain, is an interesting observation. The independence of this increase from the initiation and progression of carious lesions in these studies indicates that the increase is an inherent characteristic of these strains of rats that progresses from early life to the attainment of adult size.

The variability of the salivary protease values from time to time in rats sampled frequently as well as the variability between groups suggests that there are uncontrolled variables in the production of saliva or in the collection procedure about which we need further knowledge. However, reasonable confidence can be placed in average salivary protease values for groups of appropriate numbers of subjects.

Although these data indicate the independence of salivary protease activity and caries-susceptibility, they do not in any way suggest that saliva itself is unrelated to caries-susceptibility. The many clear indications of the relationship between saliva and dental caries incidence are not in any way contraindicated by this study in which only a single parameter, salivary protease, has been investigated.

SUMMARY

Representatives of the Harvard and Hunt-Hoppert strains of caries-susceptible rats were used to determine whether gross dietary variations and the presence or absence of carious lesions influenced protease activity and protein content of the saliva. No significant correlation was observed between salivary protease values and the diet fed nor between salivary protease values and the incidence of dental caries. Salivary protease activity increased with age in both strains with a strikingly greater increase among the Hunt-Hoppert animals. No correlation was observed between diet, animal age, strain and the concentration of salivary protein.

These data are taken as further evidence that the association between salivary protease activity in the Hunt-Hoppert strains of caries-resistant and caries-susceptible rats is a chance genetic occurrence and is not indicative of a causal relationship.

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COMPARATIVE EFFECTS OF COTTONSEED OIL, FATTY ACIDS AND DIETHYLSTILBESTROL UPON CHOLINE-DEFICIENT WEANLING ALBINO RATS [1]

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INTRODUCTION

Dietary lipide is considered to be of great importance in the extent of lipide accumulation in the livers of choline-deficient rats. It has been reported that fatty livers could be produced in rats by feeding diets high in saturated lipides (Best et al., '32). Channon et al. ('42) have shown that fatty infiltration of the liver is related to the proportion of C_{14} to C_{18} saturated fatty acids in the diet and that solid unsaturated acids exert no effect. Recently, it has been shown that butter and lard caused a greater lipide infiltration of rat liver than corn oil or margarine, when choline was omitted from the diet. In addition, the solid fatty-acid fraction of butter fat caused a greater infiltration than the liquid fraction (Benton et al., '56). In a comparison of corn oil and butter fat, it was reported that only 0.12% of dietary choline was necessary to prevent liver infiltration, if corn oil was the dietary lipide; but 0.15% of choline was required when butter fat was fed. However, the growth rate was lower on corn oil, and it appeared that there were more kidney lesions in the rats receiving corn oil (Benton et al., '57).

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This work deals with the effects of refined cottonseed oil [2] and various fatty acids upon shipped, weanling rats [3] receiving a choline-deficient diet which consisted of an adequate quantity (20%) of a low-methionine protein and a relatively low quantity (5%) of lipide.

EXPERIMENTAL

On arrival, the weanling rats were placed in individual cages, and both tap water and the experimental diets were provided ad libitum. At least 4, and in most cases, 5 animals of each sex were fed each experiment diet. The composition of the individual diets is shown in table 1.

The choline-deficient rats exhibited signs of distress between 8 and 12 days after diet initiation. The animals that died during this period are hereafter referred to as "fatalities." Equal numbers of choline-supplemented rats were killed at 10 days, and these animals will be referred to as "sacrifices." Those choline-deficient rats that survived the critical period were killed at the end of 42 days. This third group of rats will be called "choline-deficient survivors." Likewise, choline-supplemented animals were killed at the end of 42 days and will be called "choline-supplemented survivors."

The livers and kidneys were removed from all animals and stored at −25°C for analyses. Each liver was diced into small pieces about 3 mm [3]) and spread on the inside of an alundum extraction thimble (30 mm × 80 mm, medium grade). The liver was then extracted in a Soxhlet extractor with absolute ethanol for 5 siphonings and finally with petroleum ether (bp 30 to 60°C) for two hours. The solvents were removed under reduced pressure in a nitrogen atmosphere with the aid of a warm water bath (about 50°C). After solvent removal, the residue was extracted with petroleum ether (bp 60 to 71°C). The extract was then filtered, stored overnight at

[2] Wesson Oil.

[3] Sprague-Dawley, Inc., Madison, Wisconsin.

TABLE 1

Percentage composition of diets

INGREDIENT	GROUP										
	A	B	C	D	E	F	G	H	J	K	L
Soy protein [1]	20	20	20	20	20	20	20	20	20	20	20
Sucrose	68	68	68	68	68	73	68.5	68	68	68	68
Salt mix — U.S.P. XIV	4	4	4	4	4	4	4	4	4	4	4
Cellulose [2]	3	3	3	3	3	3	5.5	3	3	3	3
Linoleic acid [3]	5	2	2	2	—	—	—	—	—	—	—
Sodium butyrate [4]	—	3	—	—	—	—	—	—	—	—	—
Lauric acid [5]	—	—	3	—	—	—	—	—	—	—	—
Stearic acid [5]	—	—	—	3	—	—	—	—	—	—	—
Hydrolyzed cottonseed oil	—	—	—	—	5	—	—	—	—	—	—
Cottonseed oil	—	—	—	—	—	—	2	5	5	5	5
Diethylstilbestrol [6]	—	—	—	—	—	—	—	—	0.1	0.1 [7]	0.001

[1] Sodium Soybean Proteinate, Archer Daniel Midland, Minneapolis, Minnesota.

[2] Including the following vitamins (mg/kg diet): Thiamine·HCl, 5; riboflavin, 5; calcium pantothenate, 10; niacin, 50; pyridoxine, 10; *p*-aminobenzoic acid, 50; inositol, 250; biotin, 0.2; folic acid, 0.2; menadione, 1; choline (when present), 1500. Vitamin A alcohol, calciferol and tocopherol were dissolved in 50% ethanol and administered *per os*.

[3] 60% linoleic acid; Nutritional Biochemicals Corp., Cleveland, Ohio. The alkali isomerization method for determination of polyunsaturated acids has shown that the preparation contains 25.8% monoenoic, 59.8% dienoic, 4.3% trienoic and 0.1% tetraenoic acids.

[4] Synthesized from Butyric Acid; Nutritional Biochemicals Corp.

[5] Nutritional Biochemicals Corp.

[6] U. S. P., Merck & Co., Inc.; added to the diets in 95% ethanol solution.

[7] Withdrawn after two weeks.

—25°C and refiltered. Total dry weight was found by the gravimetric determinations of the material remaining in the extraction thimble, of the solids which were removed from the petroleum ether by filtration and, finally, of the total lipide. Total lipide was determined from an aliquot of the petroleum-ether extract which had been diluted to volume. The remaining lipide solution was stored under nitrogen at —25°C. When this method was used, it was found to have a precision of 23/1000.

The peripheral fat and membranous tunica of each kidney were removed with forceps. Then each pair was diced as were the livers and placed in an alundum extraction thimble (20 mm × 80 mm; medium grade). The kidneys were extracted for 24 hours with 95% ethanol in a Goldfisch extractor. After removal of the solvent by the use of a steam bath and ventilating hood, the residue was extracted with petroleum ether (bp 60 to 71°C), filtered into weighed cups, and the total lipide was determined gravimetrically. Total dry weight was then determined in the same manner as for liver. Complete extraction of kidney lipide by 95% ethanol was shown by the absence of any lipide after an additional 24-hour extraction with petroleum ether (bp 30 to 60°C). The lipide percentages for both the kidneys and livers were expressed on a dry-weight basis.

Iodine values were obtained by the method of Byrne and Johnson ('56). Good agreement was found between this method and the Hanus method, if chloroform was used as the lipide solvent and a two-hour reaction time was used. Phosphorus was determined by the micro-procedure of Chen and co-workers ('56).

Statistical values reported throughout this paper were determined by outlined procedures (Li, '57).

RESULTS AND DISCUSSION

Figure 1 gives the percent mortality at 8 to 12 days of both male and female rats fed different types and amounts of

lipides in the choline-deficient diets. It should be emphasized that the mortality rates observed in these experiments are demonstrable only with shipped rats, in agreement with the report of Jernstrom and King ('53). By comparing the results obtained with a 5% level of the commercial fatty acids (groups A, B, C, D) with that obtained with 5% cottonseed oil (group H), it was found that the mortality of female rats was significantly reduced ($\chi^2 = 12.1$ with 1 d.f.) by feeding the fatty acids. Numerically, the mortality rate of female rats fed hydrolyzed cottonseed oil at the 5% level (group E) was less than in the group receiving 5% cottonseed oil (group H) and greater than in the groups receiving commerical fatty acids (groups A, B, C, D), but the differences were not significant. Feeding highly unsaturated fatty acids at the 5% level (group A) significantly decreased the mortality rate of the male rats ($\chi^2 = 4.3$ with 1 d. f.) when compared with the feeding of 5% cottonseed oil (group H).

Since it is known that there are great differences in the rate and extent of absorption of a fatty acid, depending upon the presence of other fatty acids or glycerides (Bergstrom and Bergstrom, '55) dietary lipide was omitted in one experiment, and 2% cottonseed oil was fed in another. When the level of cottonseed oil was decreased from 5 to 2% (group G), the mortality of female rats was identical to that of the females which received the commercial fatty acids (groups A, B, C, D). Therefore, it would appear that at least part of the decrease in mortality in female rats fed fatty acids is due to a low absorption rate. However, if no lipide was added to the diet (group F), the female mortality rate was not significantly different from that of the 5% cottonseed oil group (group H), but was significantly higher ($\chi^2 = 4.4$ with 1 d.f.) than that of the fatty acid groups (groups A, B, C, D). This may mean that a choline deficiency increases the immediate requirement for the essential fatty acids. In addition, it is known that young rats on choline-deficient diets are prone to hemorrhagic degeneration of the kidneys within 10 days (Griffith and Wade, '39) and that a spontaneous partial recovery from kidney

damage occurs in animals that survive the initial 10-day period (Griffith, '41). These facts suggest that the presence of a minimal amount of unsaturated fatty acids protects female rats during the period in which kidney damage occurs.

The average values of the percentage of liver lipide present in all of the "fatalities" are shown in figure 2. Also the range of liver lipide of all of the "sacrifices" (both male and fe-

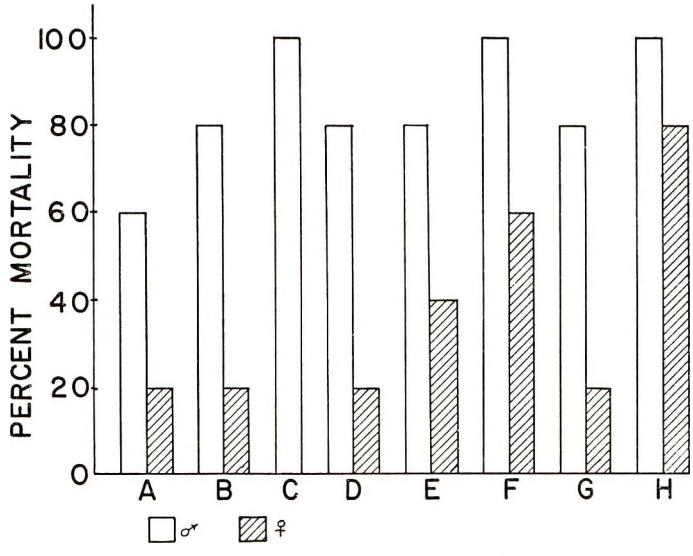

Fig. 1 Mortality rates of both sexes on various lipide diets deficient in choline. A: 5% linoleic acid; B: 2% linoleic + 3% sodium butyrate; C: 2% linoleic + 3% lauric acid; D: 2% linoleic + 3% stearic acid; E: 5% hydrolyzed cottonseed oil; F: no added lipide; G: 2% cottonseed oil; H: 5% cottonseed oil.

male) receiving the various types of dietary lipides is shown in this graph. It was previously mentioned that groups A, B, C, D and G had the lowest female mortality rates. It can be noted from figure 2 that these same groups showed significantly ($F = 20.1$ with 1,47 d.f.) less liver lipide, percentage wise, than did the others.

It was also determined that both the male and female "fatalities" of groups A, B, C, D and G had significantly more liver lipide, percentagewise, than the corresponding

choline-supplemented "sacrifices" ($F = 100.3$ with 1,42 d.f.). Thus, fatty infiltration of the liver was evident by the 12th day after diet initiation.

Figure 3 shows the liver-lipide percentages of the "choline-deficient survivors." The range of liver lipide of all "choline-supplemented survivors" is also given. Highly unsaturated fatty acids (groups A, B) were significantly more effec-

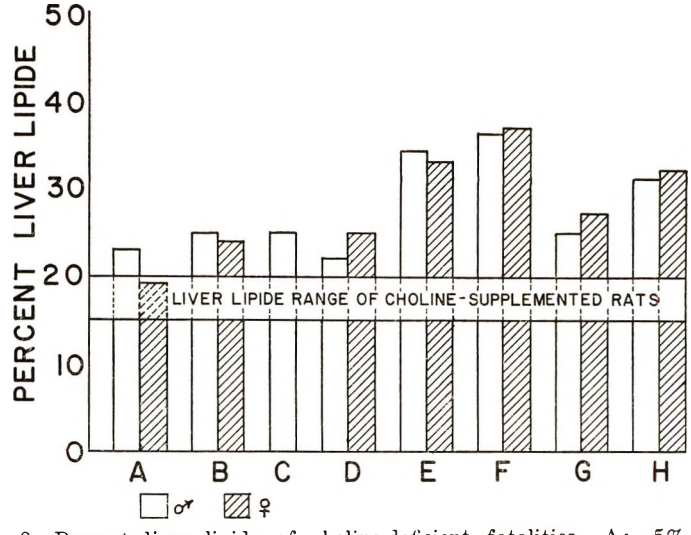

Fig. 2 Percent liver lipide of choline-deficient fatalities. A: 5% linoleic acid; B: 2% linoleic + 3% sodium butyrate; C: 2% linoleic + 3% lauric acid; D: 2% linoleic + 3% stearic acid; E: 5% hydrolyzed cottonseed oil; F: no added lipide; G: 2% cottonseed oil; H: 5% cottonseed oil.

tive ($F = 108.1$ with 1, 31 d.f.) in decreasing the amount of accumulated liver lipide than the other lipide groups (C, D, E, F, G, H). Group B can be assumed to be essentially 2% linoleic acid, since it has been found that the ethyl esters of fatty acids of less than 12 carbon atoms were ineffective in producing severe fatty livers (Stetten et al., '45). The addition of long-chain, saturated fatty acids to linoleic acid (groups C, D) resulted in significantly increased liver-lipide accumulation ($F = 65.6$ with 1, 19 d.f.).

Table 2 shows the average kidney weight and lipide values of the "fatalities," "sacrifices," "choline-deficient survivors" and "choline-supplemented survivors." All of the individual values of each of the 4 sets of animals were composited, since there were no significant differences between sexes or among dietary groups. From the table it is apparent that the "fatalities" had heavier kidneys but a smaller percentage

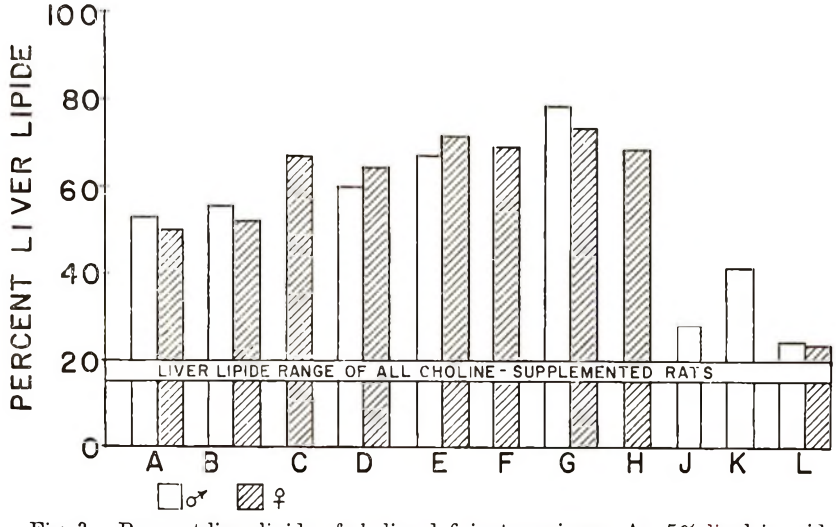

Fig. 3 Per cent liver lipide of choline-deficient survivors. A: 5% linoleic acid; B: 2% linoleic + 3% sodium butyrate; C: 2% linoleic + 3% lauric acid; D: 2% linoleic + 3% stearic acid; E: 5% hydrolyzed cottonseed oil; F: no added lipide; G: 2% cottonseed oil; H: 5% cottonseed oil; J: 5% cottonseed oil + 1000 p.p.m. diethylstilbestrol; K: 5% cottonseed oil + 1000 p.p.m. diethylstilbestrol (withdrawn); L: 5% cottonseed oil + 10 p.p.m. diethylstilbestrol.

of kidney lipide than did the "sacrifices." Also, total kidney lipide (dry weight × per cent lipide) was significantly ($F = 18.7$ with 1,87 d.f.) higher in the "fatalities." Since the "fatalities" always had a lower body weight than the "sacrifices," it would not be expected that the "fatalities" would have heavier kidneys. However, the kidneys of the "fatalities" did show signs of severe hemorrhage which might explain the unexpected weights.

There was no significant difference between the kidney lipide percentages of "choline-deficient" and "choline-supplemented survivors," but the "choline-supplemented survivors" had heavier kidneys. Accordingly, the total kidney lipide of the latter rats was significantly higher than that of the "choline-deficient survivors" ($F = 55.2$ with 1, 63 d.f.). This is to be expected, since the choline-supplemented group had a larger average body weight than did the choline-deficient group.

Since there was a significant sex difference in resistance to choline deficiency, it was decided to incorporate diethylstil-

TABLE 2

Kidney data

GROUP	DRY WEIGHT	LIPIDE	TOTAL LIPIDE
	gm	*%*	*mg*
Sacrifices	0.16 ± 0.02 [1]	28 ± 2.9	42.6 ± 6.5
Fatalities	0.26 ± 0.05	19 ± 2.5	49.4 ± 9.3
Choline-supplemented survivors	0.29 ± 0.05	27 ± 1.5	78.3 ± 13
Choline-deficient survivors	0.22 ± 0.03	26 ± 2.2	57.2 ± 7.2

[1] Standard deviations.

bestrol (DES) into the diet. Four males on a choline-deficient diet containing 5% of cottonseed oil received DES at a dietary level of 1000 p.p.m. At the end of two weeks, two of the males were removed from the DES diet and placed on an estrogen-free diet for the remaining 4 weeks. Two males and two females were placed on a similar diet containing only 10 p.p.m. of DES. All 8 rats survived, and it was the *only* time that any choline-deficient male rat receiving 5% cottonseed oil survived.

All "choline-deficient survivors" had shown a very definite growth-rate depression during the 8- to 12-day period. However, figure 4 shows that this depression did not occur if DES was present in the diet. Also, peripheral kidney calcification, which occurred in most other "choline-deficient survivors"

was not evident in any of the "choline-deficient survivors" that received DES.

Figure 3 illustrates the potent lipotropic action of DES. Male and female rats receiving DES throughout the 42-day period (groups J, L) accumulated considerably less liver lipide than did female rats that did not receive the synthetic estrogen (group H). In addition, the feeding of DES during the first two weeks and then withdrawing it for the following 4 weeks (group K) caused significantly ($F = 39.2$ with 1, 2

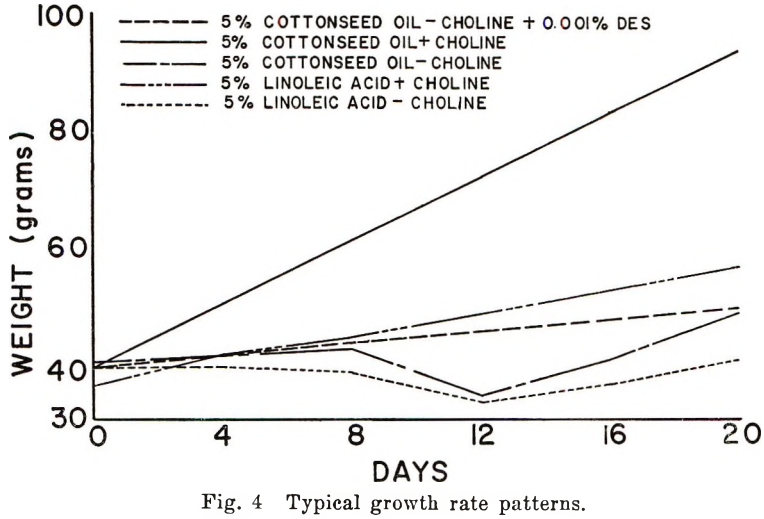

Fig. 4 Typical growth rate patterns.

d.f.) increased liver lipide accumulation when compared with that of the group receiving DES continously (group J).

It is evident then that DES, either directly or indirectly, has a lipotropic action upon choline-deficient rats. Also, the estrogen is very effective in preventing the kidney damage that occurs shortly after a choline deficiency is initiated. This latter effect may be due to the lipotropic action of the estrogen.

Tables 3 and 4 contain the lipide-phosphorus values and iodine values obtained from analysis of the liver lipide. The liver lipides extracted from each male and from each female of the "fatalities" were pooled by dietary groups for the de-

TABLE 3

Liver lipide phosphorus values

GROUP	DIETARY LIPIDE	SACRIFICES	FATALITIES	CHOLINE SUPPLEMENTED SURVIVORS	CHOLINE DEFICIENT SURVIVORS
A	5% linoleic acid	1.95	0.90	1.99	0.41
B	2% linoleic plus 3% sodium butyrate	—	1.01	—	0.45
C	2% linoleic plus 3% lauric acid	1.49	1.10	2.10	0.30
D	2% linoleic plus 3% stearic acid	1.57	1.07	1.99	0.31
E	5% hydrolyzed cottonseed oil	1.77	0.91	1.82	0.17
F	No added lipide	1.75	0.96	1.91	0.19
G	2% cottonseed oil	2.13	1.33	1.80	0.11
H	5% cottonseed oil	1.79	0.89	2.14	0.14
J	5% cottonseed oil + 0.1% DES [1]	—	—	—	1.25
K	5% cottonseed oil + 0.1% DES [2]	—	—	—	0.35
L	5% cottonseed oil + 0.001% DES	—	—	—	1.30

[1] Diethylstilbestrol.
[2] DES withdrawn.

termination of these values. The same type of pooling was used with the "sacrifices." An individual lipide-phosphorus value and an iodine value of each male and of each female survivor (both choline-deficient and choline-supplemented) were determined. However, there was no significant difference in the values between sexes of the "survivors," so the composite values of both males and females appear in the tables.

In table 3 it can be seen that the liver lipide of the "fatalities" always had a lower percentage of phosphorus than did the liver lipide of the "sacrifices." Since it has already been noted that infiltration had occurred by the 12th day, then the lipide that did infiltrate must not have contained any phosphorus. This agrees with the observation of Channon et al. ('37). The lack of phosphorus in the infiltrated lipide is even more apparent, if the lipide phosphorus values of "choline-deficient survivors" are compared with those of the "choline-supplemented survivors."

From the same table it is apparent that the presence of a continuous supply of DES to choline-deficient animals during the experimental period (groups J, L) caused an increase in the percentage of lipide phosphorus in comparison with that of animals not receiving DES or choline (group H). Since it has been mentioned previously that the presence of DES caused a considerable decrease in liver lipide accumulation, it is suggested that the lipotropic action of DES (direct or indirect) is concerned mainly with non-phosphorus-containing lipide.

Table 4, which lists iodine values, shows some interesting relationships between dietary and liver-lipide unsaturation. First, it is evident that if the dietary lipide is moderately or highly unsaturated (groups A, E, G, H), then the accumulated liver lipide of choline-deficient rats will be more unsaturated than the liver lipide of choline-supplemented rats. However, if highly unsaturated lipide is diluted with long-chain fatty acid so that the lipide is low in unsaturation (groups C, D) and then fed, the accumulated liver lipide of choline-deficient

TABLE 4

Liver lipide iodine values

GROUP	DIETARY LIPIDE	IODINE VALUES				
		Dietary lipide	Sacrifices	Fatalities	Choline supplemented survivors	Choline deficient survivors
A	5% linoleic acid	144	79	97	86	94
B	2% linoleic plus 3% sodium butyrate	58 [1]	—	82	—	86
C	2% linoleic plus 3% lauric acid	58 [1]	65	75	72	75
D	2% linoleic plus 3% stearic acid	58 [1]	69	72	70	74
E	5% hydrolyzed cottonseed oil	104 [2]	68	107	64	101
F	No added lipide	0	61	76	57	60
G	2% cottonseed oil	104	71	80	65	88
H	5% cottonseed oil	104	71	102	73	102
J	5% cottonseed oil + 0.1% DES [3]	104	—	—	—	76
K	5% cottonseed oil + 0.1% DES [4]	104	—	—	—	85
L	5% cottonseed oil + 0.001% DES	104	—	—	—	77

[1] Calculated.

[2] Minute glycerol effect neglected.

[3] Diethylstilbestrol.

[4] DES withdrawn.

rats does not become more saturated than that of choline-supplemented rats but, in fact, appears to be slightly more unsaturated. Omitting dietary lipide does not cause any consistent change in unsaturation of liver lipide.

As can be seen in figure 3 liver lipide accumulation was quite extensive in the "choline-deficient survivors" receiving 5% of hydrolyzed cottonseed oil, 2% of cottonseed oil and 5% of cottonseed oil (groups E, G, H). In table 4 it is evident that the liver lipide unsaturation of the same three groups approaches that of the dietary lipide. On the other hand figure 3 shows that 5% of linoleic acid (group A) decreased liver lipide accumulation, and in table 4 it can be noted that the liver lipide unsaturation was not nearly so high as that of the dietary lipide. These facts would suggest that the iodine value of the liver lipide is proportional to the extent of liver lipide accumulation under the above dietary conditions. This is more evident, when it is seen in figure 3 that DES (groups J, L) decreased liver lipide accumulation and also decreased liver lipide unsaturation (table 4).

A comparison of the liver lipide iodine values of "fatalities" and "sacrifices" in table 4 indicates that the unsaturation changes noted in the previous paragraphs occurred early in the experimental period.

SUMMARY

Replacing 5% of cottonseed oil in a choline-deficient diet with 5% of commerical fatty acids, either high or low in unsaturation, or with 2% of cottonseed oil, significantly reduced the mortality rate of female rats. The mortality of female rats receiving a choline-deficient diet containing no added lipide was approximately equivalent to that of rats receiving 5% of cottonseed oil. The commercial fatty acids and 2% cottonseed oil diets also significantly decreased liver-lipide accumulation 8 to 12 days after diet initiation in comparison with results obtained with the 5% cottonseed oil diet. A commercial linoleic acid preparation was significantly more

effective than any other lipide in reducing liver-lipide accumulation 42 days after diet initiation.

Diethylstilbestrol provided complete protection against death to both males and females fed a choline-deficient diet even if it was fed at a level of 10 p.p.m. The growth depression which occurred in choline-deficient rats 8 to 12 days after diet initiation was prevented by diethylstilbestrol. The synthetic estrogen also significantly reduced liver lipide accumulation.

Feeding moderately and highly unsaturated lipide to choline-deficient rats caused an increase in the unsaturation of liver lipide. The unsaturation increase appeared to be proportional to the extent of liver-lipide accumulation. Feeding highly saturated lipide did not decrease the unsaturation of liver lipide even though accumulation was extensive.

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THE AMINO ACID REQUIREMENTS FOR MAINTENANCE IN THE ADULT ROOSTER [1]

I. NITROGEN AND ENERGY REQUIREMENTS IN NORMAL AND PROTEIN-DEPLETED ANIMALS RECEIVING WHOLE EGG PROTEIN AND AMINO ACID DIETS

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Little information is available concerning the maintenance requirements for amino acids in the adult chicken. The development of a free amino acid diet for the laying hen which maintains egg production (Fisher and Johnson, '56) made it possible to define the amino acids essential for egg production (Johnson and Fisher, '56) and to determine minimal requirements for these amino acids (Johnson and Fisher, '58).

In a recent study Ariyoshi ('57) determined the minimum amount of nitrogen required for the maintenance of nitrogen equilibrium in adult roosters depleted of their protein reserves, using whole egg protein as the nitrogen source. In determining this requirement it was assumed that nitrogen utilization in the protein-depleted animal does not differ from utilization in non-depleted animals. Evidence that this assumption may not be correct has been presented by Allison

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('51) who has stressed the importance of the body protein reserves in interpreting nitrogen balance data.

The importance of the protein stores of the animal is further emphasized by the studies of Nasset ('57) who determined the requirement for each of the essential amino acids for maintenance of nitrogen equilibrium in rats depleted of their protein reserves; when all of the essential amino acids were fed at the previously determined level, nitrogen equilibrium could not be maintained.

The present studies were undertaken in an attempt to develop a free amino acid diet supplying minimal levels of nitrogen for nitrogen equilibrium which could be used to study the maintenance requirement for the essential amino acids in the adult rooster. The nitrogen requirement was studied with whole egg protein and free amino acids as nitrogen sources in protein-depleted and non-depleted roosters. The minimum energy requirement was also ascertained on the amino acid diet since Rose et al. ('54) demonstrated a higher energy requirement on free amino acid diets as compared with protein-containing diets.

EXPERIMENTAL AND RESULTS

Mature White Leghorn roosters were used in all studies. The animals were maintained in cages in a temperature regulated room (65 to 72°F) and received a stock mash diet when not on experiment. The number of animals fed on each test diet is indicated in the tables of results.

In table 1 are shown a free amino acid and a whole egg protein diet typical of the diets used throughout these studies. To obtain the different energy and nitrogen levels studied, adjustments were made with amino acids or whole egg protein, equal parts of glucose and dextrin, and with corn oil.

The experimental procedure with birds not previously depleted of protein was to feed the basal diet containing 7.06% whole egg protein for a one-week period prior to the start of the test period. This diet was fed in the amount of 25 gm/kg body weight/day and supplied adequate amounts of nitrogen

and energy (282 mg nitrogen and 90 Cal. of metabolizable energy [2] kg/day) to maintain positive nitrogen balance. This adjustment period was considered important from the standpoint of standardizing the level of protein reserves in the experimental animal.

TABLE 1

Composition of experimental rations

INGREDIENT	AMOUNT	
	Whole egg diet [1]	Amino acid diet [2]
	%	%
Dried whole egg (44.12% protein)	16.00	—
Amino acid mixture [3]	—	8.93
Dextrin	37.78	34.81
Cerelose	37.77	34.81
Fiber	4.00	4.00
Mineral mix [4]	3.00	3.00
Corn oil	1.00	12.00
B-vitamin mix [4]	0.15	0.15
Vitamin A, D, E mix [4]	0.10	0.10
Choline chloride	0.20	0.20
Antiacid adsorbent [4]	—	1.00
Sodium bicarbonate	—	0.50
Potassium bicarbonate	—	0.50
Total	100.00	100.00

[1] Diet fed to group 3, table 2 at the rate of 25 gm/kg body wt.

[2] Diet fed to group 1, table 3 at the rate of 24 gm/kg body wt.

[3] Amino acid mixture contained the following as a percentage of the diet: L-arginine·HCl, 0.61; L-histidine·HCl·H_2O, 0.21; L-lysine HCl (99% purity), 0.61; L-tyrosine, 0.32; L-tryptophan, 0.10; DL-phenylalanine, 0.85; L-cystine, 0.18; DL-methionine, 0.28; DL-threonine, 0.62; L-leucine, 0.69; DL-isoleucine, 1.10; DL-valine, 1.06; L-glutamic acid, 0.92; and glycine, 1.38.

[4] Fisher and Johnson ('56).

Following the standardization period, the birds received the test diets for an equilibration period of three days before determinations of nitrogen balance were initiated. Fritz et al. ('36) have shown that a three-day period is sufficient to overcome any effect of the previous diet on nitrogen excretion in

[2] Calculated from data of Anderson ('55).

the adult rooster. Daily nitrogen balance was then determined during a subsequent three-day period. The values presented in the tables represent the average balances carried out on three consecutive days for each bird on each test diet; since 4 birds were usually used per treatment each value represents an average of 12 determinations.

All diets were force-fed during the balance periods in two separate feedings at 9 and 11 A.M. The diets were mixed in a blender with sufficient water to obtain the consistency of a heavy cream. The mixture was then fed by means of a 50-ml syringe to which was attached a length of 3/8 inch polyethylene tubing which extended into the crop of the animal. The feed mixture was fed quantitatively by weighing the polyethylene tubing and syringe, before and after delivery into the crop.

Initially, difficulties were encountered with the force-feeding procedure. The birds, after being fed, would drink large quantities of water and would regurgitate some of the diet. This difficulty was overcome by permitting the animals access to water from 4 P.M. to 8 A.M.

The mixed excreta (urine and feces) were collected for 24-hour periods, homogenized with water in a blender to which were added a few drops of an antifoam agent [3] and finally diluted to 500 ml. Aliquots of 5 ml were then taken by pipette for nitrogen analysis. Nitrogen in the excreta and feed was determined by a semimicro Kjeldahl method. The small differences between calculated and actual nitrogen content of experimental rations are indicated in the respective tables of results.

The first series of experiments was designed to determine the nitrogen requirement for the maintenance of nitrogen equilibrium in roosters not previously depleted of protein. Whole egg protein was first used as the nitrogen source and was supplied at three levels of nitrogen: 215, 250 and 282 mg/kg/day. Each nitrogen level was provided at a constant Cal-

[3] Dow-Corning antifoam B, courtesy of Dow-Corning Corp., Midland, Michigan.

oric intake, calculated at 90 Cal. of metabolizable energy/kg/day. The data in table 2 indicate that the level of 215 mg/kg was inadequate since a severe negative nitrogen balance resulted. The next higher level of nitrogen intake, 250 mg/kg/day, was still insufficient to maintain the birds in positive nitrogen balance. The birds were in positive nitrogen balance

TABLE 2

Nitrogen requirement from whole egg protein for maintenance of nitrogen equilibrium in mature roosters

GROUP	1	2	3
Calculated mg N/kg body wt./day	215	250	280
Calculated Cal./kg body wt./day [1]	90	90	90
Average initial body wt.; gm	2025	2130	2120
Number of animals	4	4	3
Nitrogen balance data	(mg N/kg body wt./day)		
N intake	215	250	282
N excretion	556 ± 82 [2]	326 ± 43	205 ± 15
N balance	— 341	— 76	+ 77
Δ body wt., gm	— 20	— 20	— 5

[1] Calories of metabolizable energy.
[2] Standard error of mean.

TABLE 3

Energy requirement of mature roosters receiving free amino acid diets formulated to simulate the amino acid composition of whole egg protein

GROUP	1	2	3	4
Calculated mg N/kg body wt./day	280	280	280	280
Calculated Cal./kg body wt./day [1]	90	100	110	115
Average initial body wt., gm	2020	1955	1930	2110
Number of animals	3	4	4	4
Nitrogen balance data	(mg N/kg body wt./day)			
N intake	274	286	283	270
N excretion	318 ± 23 [2]	323 ± 9	248 ± 8	259 ± 12
N balance	— 44	— 46	+ 35	+ 11
Δ body wt., gm	— 60	— 25	+ 60	— 15

[1] Calories of metabolizable energy.
[2] Standard error of mean.

when the nitrogen intake was further increased to 282 mg/kg/day.

The data presented in table 3 demonstrate the increased energy requirement on a free amino acid diet. The nitrogen portion of the diet was formulated to simulate whole egg protein in amino acid composition in such a way that the L-isomers of 12 of the amino acids were supplied at the same

TABLE 4

Amino acids supplied in whole egg protein and free amino acid diets per 280 mg N [1]

WHOLE EGG PROTEIN DIET		FREE AMINO ACID DIET	
Amino acid	mg supplied/kg/day	Amino acid	mg supplied/kg/day
Arginine	121.4	L-Arginine·HCl	146.3
Histidine	37.0	L-Histidine HCl·H_2O	50.0
Lysine	116.8	L-Lysine HCl (99%)	147.8
Tyrosine	77.4	L-Tyrosine	77.4
Tryptophan	24.6	L-Tryptophan	24.6
Phenylalanine	102.1	DL-Phenylalanine	204.2
Cystine	42.2	L-Cystine	42.2
Methionine	70.7	DL-Methionine	70.7
Threonine	73.9	DL-Threonine	147.8
Leucine	165.4	L-Leucine	165.4
Isoleucine	132.0	DL-Isoleucine	264.0
Valine	126.7	DL-Valine	253.4
Glutamic acid	221.8	L-Glutamic acid	221.8
		Glycine	301.1

[1] Amino acids present in whole egg protein calculated from data of Block and Weiss ('56).

level as found in an amount of whole egg protein represented by 280 mg of nitrogen; the remainder of the nitrogen was supplied by the D-amino acids used (phenylalanine, threonine, isoleucine and valine) and by glycine (table 4). It was assumed that the D- and L- isomers of methionine were equally available to the bird. Metabolizable energy levels of 90, 100, 110 and 115 Cal./kg/day were tested. The 90 Cal./kg/day level which had maintained a positive nitrogen balance when the

same amount of nitrogen was supplied from whole egg protein, gave rise to a slightly negative nitrogen balance. Only when the Caloric intake was increased to 110 or 115 Cal./kg was positive nitrogen equilibrium maintained on the free amino acid diet.

The experiment presented in table 5 was designed to determine the nitrogen requirement on the free amino acid diet when only the level of glycine was varied. The levels of the

TABLE 5

Nitrogen requirement of adult roosters receiving free amino acid diets formulated to simulate the essential amino acid composition of whole egg protein

GROUP	1	2	3
Calculated mg N/kg body wt./day [1]	225	250	280
Calculated Cal./kg body wt./day [2]	120	120	115
Average initial body wt., gm	1965	2050	2110
Number of animals	4	4	4
Nitrogen balance data	(mg N/kg body wt./day)		
N intake	236	257	270
N excretion	307 ± 29 [3]	268 ± 22	259 ± 12
N balance	— 71	— 11	+ 11
Δ body wt., gm	— 10	— 30	— 15

[1] Nitrogen content varied by increasing the amount of glycine only.
[2] Calories of metabolizable energy.
[3] Standard error of mean.

other 13 amino acids presented in table 4 remained constant and the diets supplied essentially the same Caloric intake previously determined as adequate. An intake of 236 mg N/kg proved inadequate and resulted in a negative nitrogen balance. When the nitrogen intake was increased to 257 or 270 mg/kg nitrogen equilibrium was attained. These data show a nitrogen requirement greater than that supplied by the essential amino acids.

The next experiments were designed to study the nitrogen requirements on whole egg protein and free amino acid diets

in protein-depleted animals. In figure 1 the nitrogen excretion on a nitrogen-free diet is plotted against time. It can be seen that the nitrogen excretion reached a plateau after three days on the nitrogen-free diet; this was considered to be the endo-

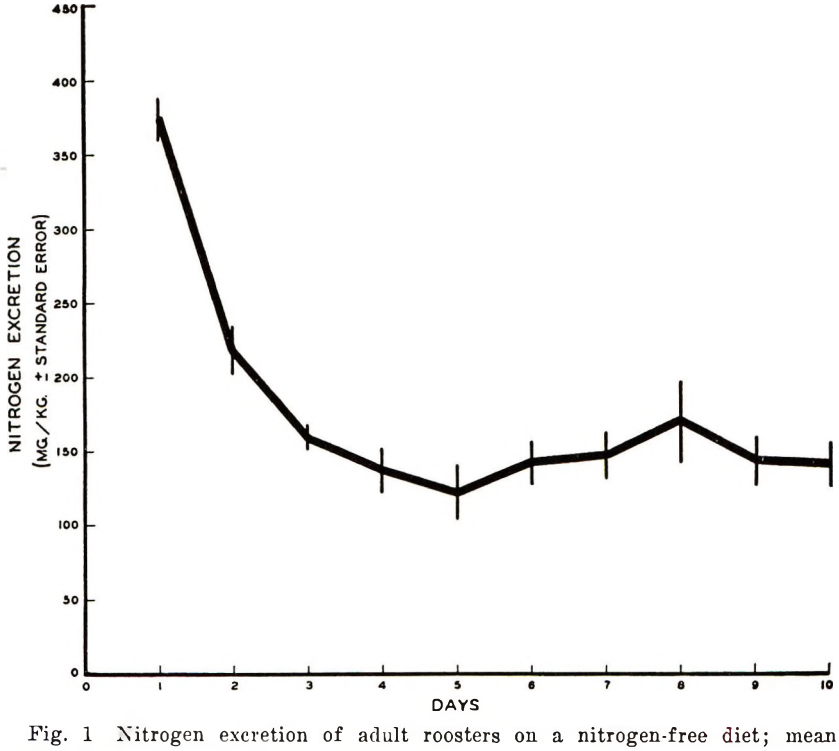

Fig. 1 Nitrogen excretion of adult roosters on a nitrogen-free diet; mean values for 4 animals. Initial average body weight was 2215 gm, weight loss during the 10 day experimental period was 7% of the initial weight.

genous nitrogen excretion. The mean value (± standard error) for the last 7 days on the diet was 143 ± 6 mg/kg/day. Following a one week depletion period, birds were fed a whole egg protein and a free amino acid diet which were calculated to supply only half of the previously determined nitrogen requirement (140 mg/kg/day) and which therefore supplied only half of the amounts of each of the amino acids listed in

table 4. The results of this trial are presented in table 6. It can be seen that 140 mg/kg/day from either whole egg protein or free amino acids essentially maintained nitrogen equilibrium. From these data it is evident that the depleted animal exhibits an altered nitrogen metabolism as compared to the non-depleted animal since it can maintain nitrogen equilibrium with only half the nitrogen required by the non-depleted individual.

TABLE 6

Nitrogen requirement from whole egg protein and a free amino acid mixture for maintenance of nitrogen balance in the protein-depleted adult rooster

NITROGEN SOURCE	WHOLE EGG PROTEIN	FREE AMINO ACIDS
Calculated mg N/kg body wt./day	140	140
Calculated Cal./kg body wt./day [1]	99	126
Average initial body wt., gm	1970	1975
Number of animals	4	4
Nitrogen balance data	(mg N/kg body wt./day)	
N intake	146	148
N excretion	165 ± 12 [2]	156 ± 9
N balance	— 19	— 9
Δ body wt., gm	+ 15	+ 15

[1] Calories of metabolizable energy.
[2] Standard error of mean.

DISCUSSION

A protein of high biological value is essential to the study of the minimal nitrogen requirement for maintenance. However, such a nitrogen source does not permit the separate determination of a requirement for non-essential and essential amino acid nitrogen. In the present study this difficulty has been overcome by simultaneously studying the nitrogen requirement with whole egg protein and with an amino acid mixture formulated to simulate whole egg protein in essential amino acid composition. Under these conditions it was possible to demonstrate a requirement for non-essential nitrogen.

Since the total nitrogen requirement was similar with both nitrogen sources the balance of essential to non-essential nitrogen in whole egg protein appears to be optimal as would be anticipated from its high biological value.

The nitrogen from free amino acids has been considered less well utilized for growth (Fox et al., '58) than that from a protein source. The data presented here show that nitrogen for maintenance from free amino acids is only slightly less well utilized than that from whole egg protein in the non-depleted animal and equally well utilized in the depleted bird.

The results of the present study further illustrate the importance of considering the protein reserve status of the animal in interpreting nitrogen balance data (Allison, '51).

Ariyoshi ('57) reported a requirement of approximately 150 mg N/kg/day for maintenance purposes. This requirement is similar to that found in the present study for the protein-depleted rooster, although it is only half of the requirement determined for the non-depleted animal.

The endogenous nitrogen excretion observed in the present study (143 mg/kg/day) agrees well with the value of 144 mg/kg/day determined by Ackerson et al. ('26) for the non-molting hen. It does not agree, however, with the value of 116 mg/kg/day reported by Ariyoshi. His lower value may be attributed in part to the rather large fluctuations in body weight of his animals and to his method of analysis. In his procedure the feces were oven-dried, a practice which can lead to considerable loss of nitrogen (Obradovic, '57). The weight loss during the depletion period in Ariyoshi's study amounted in some instances to over 20% of the initial body weight while in the present study the average loss was only 7% of the initial weight.

Ariyoshi also reported that a depletion period of two months was required to reach the endogenous state while the present study, in agreement with the report of Fritz et al. ('36), indicates that a period of three days is sufficient.

The nitrogen requirement observed in the depleted bird, when expressed as a function of the basal energy expenditure

agrees well with the value of 2 mg of N/basal Calorie observed in other species (Brody, '45). The basal metabolism for a 2-kg bird determined by Brody's equation ('45) is 129 Cal./day; the N requirement for such a bird in the endogenous state would be 286 mg N/day and the nitrogen requirement therefore 2.2 mg/basal Calorie.

The experiments with varying energy levels indicate clearly that at least 20 Cal./kg/day are needed in excess on free amino acid diets compared to protein containing diets. The value of 90 Cal./kg/day for the animals receiving the protein-containing diets appears to be close to the minimal requirement. The adequacy of the energy levels used is reflected by the small weight changes observed. Miller and Allison ('58) reported a requirement of approximately 60 Cal./kg/day for the adult cat maintained in metabolism cages which was similar to the requirement for the beagle dog. Since the chicken has a higher body temperature than either of these animals, a higher caloric requirement would be anticipated.

SUMMARY

The nitrogen requirement for the maintenance of nitrogen balance in the protein-depleted and non-depleted adult male chicken has been determined. Whole egg protein and a free amino acid mixture in which 13 of the amino acids were supplied at the same level as found in whole egg protein served as nitrogen sources.

The nitrogen requirement in the non-depleted bird was found to be 280 mg N/kg/day with either whole egg protein or free amino acids as the nitrogen source. In the protein-depleted animal the nitrogen requirement was half of that of the non-depleted animal or 140 mg N/kg/day from either whole egg protein or free amino acids.

The metabolizable energy requirement at a nitrogen intake of 280 mg N/kg/day is not greater than 90 Cal./kg/day with whole egg protein as the nitrogen source. When free amino acids served as the nitrogen source the Caloric requirement

was increased by at least 20 Cal. of metabolizable energy/kg/day.

The adult rooster reached the endogenous level of nitrogen excretion after three days on a nitrogen free diet. The endogenous nitrogen excretion was 143 mg N/kg/day. The minimum nitrogen requirement based on the endogenous nitrogen excretion was 2.2 mg N/calculated basal Calorie/day.

The results of the present study illustrate the importance of the state of body protein reserves on the nitrogen requirement of the adult male chicken.

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