EFFECT OF HIGH ENVIRONMENTAL TEMPERATURES ON METABOLISM

I. GROWTH AND BLOOD CONSTITUENTS OF RATS EXPOSED TO 94°F. FOR 72 HOURS [1]

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Temperature has an effect on the metabolism and nutritional requirements of men and animals, especially in desert or tropical climates. Mills ('41, '42, '43) has claimed that rats fed ad libitum at a temperature of 91°F. need increased quantities of thiamine, choline and pyridoxine for optimum growth. Sarett and Perlzweig ('43), however, have demonstrated that, if feed intake is equalized, rats at 91°F. gain more weight and retain more nitrogen, fat and water than control groups; and Kline et al ('45) have reported that when rats are kept at a temperature of 90°F., their thiamine requirements actually decrease. This decrease was found to approximate the decrease in caloric requirement at the elevated temperature.

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This report is the first of several planned on the nutritional consequences of high environmental temperatures, both to animals and human beings. In this study rats were kept at 94°F. for 72 hours; and the response of the animals in terms of mortality rate, feed consumption, growth, and serum levels of protein, riboflavin, carotene, vitamin A, ascorbic acid, alkaline phosphatase and phosphorus was recorded.

PROCEDURE

In experiment 1, which consisted of 5 trials and extended over a 6-month period, feed was offered ad libitum and consumption was measured. In experiment 2, which consisted of 4 trials and extended over a 4-month period, feed was restricted to 5 gm and all of the feed offered was consumed. The total period covered by both experiments was 12 months. Water was supplied ad libitum in all trials.

The ration fed to all rats during each trial — and for 6 weeks preceding it — consisted, in per cent, of sesame oil meal, 40; powdered whole milk, 20; crude casein, 5; ground yellow corn, 28.4; cottonseed oil, 1; a commercial vitamin concentrate,[4] 1.6; and a mineral mixture that includes the minor elements, 4.

While the same feed formula was used throughout, the yellow corn and powdered whole milk were obtained from several sources during the two experiments and a new supply of the vitamin concentrate was obtained especially for the trials of experiment 2. Sufficient quantities of the rations were prepared in advance for each trial of both experiments, and these were stored in glass bottles in a cool room.

White male rats, weighing from 225 to 285 gm, were used. They were third-generation progeny of stock from the Bu-

[4] VitaRich. According to the manufacturer, the Thompson Hayward Chemical Co., it contains, per pound (in a carrier of sardine and whey solubles, fish liver and glandular meals, etc.), not less than 300 mg riboflavin, 250 mg pantothenic acid, 10,000 mg choline, 20 mg thiamine, 300 mg niacin, 0.5 mg vitamin B_{12}, 90,800 A.O.A.C. chick units of vitamin D and 90,800 U.S.P. units of vitamin A.

reau of Animal Industry, United States Department of Agriculture. They were assigned to experimental groups at random on the basis of age, and each group was placed in an all-wire cage. Control groups were kept in a room that had an average temperature of 76°F. and a humidity of 35 to 75%; high-temperature groups were kept in a converted Humidaire incubator, where a ventilating fan and thermostatic controls maintained an average temperature of 94°F. and a humidity of 50 to 75%.

The amount of heat used in these experiments — 94°F. — and the length of the trial period — 72 hours — were determined by preliminary trials, which indicated that higher temperature and longer periods would result in excessive mortality.

At the end of the trial period each rat was bled by heart puncture. The blood sera were analyzed by ultramicro methods for total protein (Lowry and Hunter, '45), riboflavin (Burch et al., '48), total carotenoids and vitamin A (Bessey et al., '46b), ascorbic acid (Lowry et al., '45) and alkaline phosphatase (Bessey et al., '46a). In experiment 2, phosphorus was determined in the sera by the method of Fiske and Subbarow (Hawk et al., '47).

Statistical methods outlined by Snedecor ('46) were used to analyze the data of the experiments. The "t" test was used to determine the significance of data on the effect of heat on the blood constituents; and two-way tables were used when studying the interrelationships among method of feeding and temperature and the levels of the blood constituents reported.

RESULTS

Data from experiment 1 indicate that rats kept at a high temperature for 72 hours cut down their feed intake significantly, lose much weight, and have a higher mortality rate than rats in control groups (table 1). The level of total carotenoids in their blood apparently undergoes no change, but the levels of riboflavin, vitamin A, ascorbic acid and

TABLE 1

Mortality, feed consumption, weight gain and changes in constituents of the blood sera of rats subjected to 76°F. and 94°F. for 72 hours and fed ad libitum

(Experiment 1)

TRIAL	NO. OF RATS	MORTALITY	AVE. FEED CONSUMED PER RAT	AVE. WEIGHT GAINED PER RAT	PROTEINS	RIBOFLAVIN	CAROTENOIDS	VITAMIN A	ASCORBIC ACID	ALKALINE PHOSPHATASE
			gm	*gm*	*gm/%*	*μg/%*	*μg/%*	*μg/%*	*mg/%*	*mM/l/hr.*[1]
Control groups (76°F.)										
1	9	0	33.3	10	6.54	5.66	5	26.3	1.52	5.9
2	7	0	51.0	33	6.26	1.73	4	27.1	1.02	3.9
3	9	0	38.3	19	6.92	2.68	2	19.5	1.96	9.7
4	9	1	41.8	28	6.26	3.07	2	32.8	2.16	10.5
5	5	0	23.1	10	6.56	4.31	2	22.7	0.84	...
Average[2]	..	..	37.5	20	6.51 ± 0.46	3.49 ± 1.72	3	25.7 ± 7.3	1.50 ± 0.63	7.5 ± 3.4
Heat-treated groups (94°F.)										
1	9	0	5.5	—40	7.24	3.40	4	16.2	1.07	4.2
2	8	2	9.1	— 1	6.62	1.79	6	13.4	1.37	1.5
3	9	1	4.0	—37	7.33	2.29	4	14.3	1.55	7.3
4	9	7[3]	3.1	—53	6.72	2.71	4	14.9	1.09	2.8
5	9	1	4.0	—26	7.14	2.72	2	4.9	0.62	0.8
Average[2]	..	.	5.1	—31	7.01 ± 0.48	2.58 ± 0.83	4	12.7 ± 5.6	1.14 ± 0.44	3.3 ± 2.6

[1] Millimoles per liter per hour.

[2] Over-all means and standard deviations of all the observations.

"t" test: P.= proteins, < 0.0001; riboflavin, < 0.009; vitamin A, < 0.0001; ascorbic acid, < 0.002; alkaline phosphatase, < 0.0001.

[3] High mortality was the result of high temperature: a faulty thermostat permitted a temperature of 100°F. for a brief period.

TABLE 2

Mortality, feed consumption, weight gain and changes in constituents of the blood sera of rats subjected to 76°F. and 94°F. for 72 hours and restricted in feed intake

(Experiment 2)

TRIAL	NO. OF RATS	MORTALITY	AVE. FEED CONSUMED PER RAT	AVE WEIGHT GAINED PER RAT	PROTEINS	RIBOFLAVIN	CAROTENOIDS	VITAMIN A	ASCORBIC ACID	ALKALINE PHOSPHATASE	PHOSPHORUS
			gm	*gm*	*gm/%*	*μg/%*	*μg/%*	*μg/%*	*mg/%*	*mM/l/hr.*[1]	*mg/%*
					Control groups (76°F.)						
1	9	2	5	— 9	5.88	3.15	2	17.9	2.33	7.2	..
2	9	0	5	— 28	7.40	2.77	2	17.9	1.31	5.0	2.3
3	9	0	5	— 45	6.57	3.05	2	16.9	2.63	9.6	3.3
4	9	0	5	— 37	6.84	2.30	2	16.5	2.61	10.3	3.7
Average [2]	..	..	5	— 30	6.67 ± 0.72	2.82 ± 0.81	2	17.8 ± 7.7	2.22 ± 0.62	8.0 ± 3.0	3.1 ± 0.76
					Heat-treated groups (94°F.)						
1	9	1	5	— 19	6.62	3.46	2	13.9	1.63	10.6	..
2	9	0	5	— 25	7.62	2.71	1	11.9	1.09	5.9	2.3
3	9	0	5	— 36	6.52	2.49	1	10.3	1.61	8.4	3.2
4	9	0	5	— 29	7.15	2.46	1	15.8	1.42	9.6	3.4
Average [2]	..	..	5	— 28	6.98 ± 0.71	2.78 ± 0.74	1	13.0 ± 3.7	1.43 ± 0.36	8.6 ± 3.0	3.0 ± 0.64

[1] Millimoles per liter per hour.

[2] Over-all means and standard deviations of all the observations.

"t" test: P = proteins, 0.23; riboflavin, 0.55; vitamin A, < 0.007; ascorbic acid, < 0.0001; alkaline phosphatase, 0.60; phosphorus, 0.80.

alkaline phosphatase are significantly depressed. Their total serum protein, on the other hand, reaches a significantly higher level.

In experiment 2 both the control and the high-temperature groups lost weight, the average loss being about the same for both, and the negligible difference in mortality was in favor of the high-temperature groups (table 2). As in experiment 1, the high temperature seemed to have no effect on serum carotenoids; however, the levels of vitamin A and ascorbic acid were significantly depressed. Serum riboflavin, alkaline phosphatase and phosphorus were not affected. The level of total serum protein was elevated in three of the 4 trials, but the over-all average showed no significant increase.

DISCUSSION

Since an equivalent weight loss was observed in both the control and high-temperature groups of experiment 2, it is evident that the weight losses of the rats in experiment 1, which were subjected to the same high environmental temperature, were due primarily to decreased food intake. The water consumption of the rats subjected to the high temperature was considerably more than that of the control groups. In both experiments the rats subjected to the high temperature prostrated themselves on the floors of their cages and used part of the water for external cooling.

It is apparent from these studies that a high environmental temperature (94°F.) has a depressive effect on serum riboflavin, alkaline phosphatase, ascorbic acid and vitamin A. The effect of heat in producing a depression of serum riboflavin and alkaline phosphatase in animals fed ad libitum (experiment 1) largely disappeared under conditions of restricted feed intake (experiment 2). On the other hand, serum ascorbic acid and vitamin A were significantly depressed under both conditions.

A statistical comparison of the data of experiment 1 and those of experiment 2 shows that the depressive effect of the

high temperature on serum ascorbic acid and vitamin A is the result of a direct effect of heat on these blood constituents, accompanied by an effect resulting from the restriction of feed. Differences were observed in the levels of several blood constituents between the control groups of experiment 1 and experiment 2. These cannot be attributed exclusively to food restriction, since part could have been due to the use of several sources of corn and milk powder and a new supply of the vitamin concentrate in formulating all rations. There is also the possibility that the high serum ascorbic levels observed in the control groups of experiment 2 were in part a result of the restricted feeding. The direct depressive effect of temperature on the serum ascorbic acid and vitamin A levels was over and above any net feeding effect.

The nature of this apparent effect of high temperature on vitamin A and ascorbic acid serum levels observed in these experiments is not known. Chemical analyses showed that the heat did not destroy vitamin A and ascorbic acid in the test rations; hence the intake of these two nutrients was similar for both the control and heat-treated groups. Utilization of both nutrients may have increased, or absorption of carotenoids, vitamin A, and ascorbic acid from the gastrointestinal tract may have decreased, or both. Since the carotenoids stayed at the same level in both the control and heat-treated groups, it does not seem likely that the lower levels of vitamin A in the latter were due to a poor conversion of carotene to vitamin A. The data on depression of vitamin A and ascorbic acid are consistent with findings of Kurokawa ('41), who showed that avitaminosis A could be induced more rapidly in rats in a hot environment than in a cool one, and the results of Daum et al. ('39), who demonstrated that a high body temperature may lower blood plasma C in man.

No explanation is available for the low alkaline phosphatase level observed in the heat-treated rats of experiment 1, when presumably normal levels were observed in the heat-treated animals of experiment 2.

The significant increase in the serum levels of total protein in experiment 1 and in three of the 4 trials in experiment 2 indicates that high temperature does influence serum protein. Whether one or all of the serum protein fractions were affected is not known, inasmuch as fractionation studies were not made.

It was recognized that the differences in the serum levels of protein, ascorbic acid and vitamin A observed in the rats subjected to high temperature might have disappeared or become less marked if it had been possible to extend the length of the experimental period. Preliminary studies had shown, however, that when the test period was extended to 7 days the mortalities were excessive.

The results of these experiments suggest the need for further studies on the effects of climatic stress on the metabolism of serum proteins, vitamin A and ascorbic acid in animals in desert and tropical climates. Also a possible interrelation of vitamin A and ascorbic acid, such as that reported by Mayer and Krehl ('48), should be considered.

SUMMARY

Rats were subjected to a temperature of 94°F. for 72 hours and were then compared with control rats, which had been kept at a temperature of 72°F. In one set of trials they were fed ad libitum; in another they were restricted to 5 gm of feed.

With ad libitum feeding, heat-treated rats consumed less feed than control rats. They lost weight, and the levels of riboflavin, vitamin A, ascorbic acid and alkaline phosphatase in their blood sera were significantly depressed, whereas the levels of total protein were significantly elevated.

With restricted feeding, both control and heat-treated rats lost weight, and in the heat-treated rats only the vitamin A and ascorbic acid levels were significantly depressed. The exposure to high temperature had no effect on total carotenoid levels in either the ad libitum or feed-restricted groups.

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THE OCCURRENCE OF A CALCINOSIS SYNDROME IN COTTON RATS

IV. THE EFFECT OF DIET AND THE AGE OF THE ANIMALS ON THE DEVELOPMENT OF THE DISEASE AND ON THE URINARY EXCRETION OF VARIOUS METABOLITES [1]

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SEVEN FIGURES

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INTRODUCTION

During the course of nutritional studies in which weanling cotton rats from our colony were used, we observed a diffuse calcification confined primarily to the cardiac and skeletal muscles but frequently affecting the livers of experimental animals if they were fed semi-purified diets. A survey of the effects of many types of diets on the development of this disease and the growth of the animals (Constant and Phillips, '52) and a description of the pathology of the lesions (Constant, Phillips and Angevine, '52) have been reported. Several similarities were noted between the lesions and the effect of diet on the disease in cotton rats, and in guinea pigs fed diets deficient in the anti-stiffness factor.

Since growth rate changes only partially reflected the development of the disease, and the involvement of the heart

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made routine blood samples from this source impractical, urinary analyses were made in order to find other criteria which would permit a clinical pursuit of the development of the disease. The urinary excretion levels of creatine and phosphorus were followed in each case, and such other urinary analyses and enzyme studies were made as were suggested by the data obtained, in order to gain further information on this disease. Young and adult animals were fed synthetic or stock diets to determine whether the cotton rats were more susceptible to the development of the disease during their rapid growth period. White rats were also used to compare the effects of similar diets on their urinary excretion levels of the various substances studied with the results obtained when cotton rats were used.

EXPERIMENTAL

In the experiments in which young (three- to 4-week-old) cotton rats were used, litter mates were distributed one each per lot. The white rats were weanling male rats of the Sprague-Dawley strain. The animals were kept in individual metabolism cages 6 inches in diameter and given food and water ad libitum. Haliver oil supplements were given every 8th day. The basal diet contained crude casein (24%), sucrose (67%), modified salts IV (4%) and corn oil (5%). The mineral mix provided 20 mg of Mg, 560 mg of Ca and 280 mg of P per 100 gm of ration in all experiments except the first, in which the magnesium level was 10 mg %. The following levels of B vitamins per kilogram of ration were used and are slightly higher than the amounts shown to be required by this species (Schweigert, '48): thiamine HCl, 3.8 mg; pyridoxine HCl, 3.8 mg; riboflavin, 4.5 mg; nicotinamide, 38.0 mg; calcium pantothenate, 30.0 mg; choline, 1.5 gm; inositol, 1.5 gm; *p*-aminobenzoic acid, 0.45 gm. Finely ground guinea pig pellets [2] were used as the stock diet and contained approximately 1.4% Ca, 0.5% P and 0.3% Mg.

[2] Rockland.

Urine was collected under toluene, acidified, taken to 100 ml in volume and filtered through no. 1 filter paper. Creatine and creatinine were determined by the alkaline-picrate method. In the first two experiments the conversion of creatine to creatinine was accomplished by autoclaving the samples with 1N HCl. Since it was found that autoclaving for 45 minutes with picric acid gave more uniform and reproducible results, this procedure was used in the remainder of the experiments. Phosphorus was determined by the Fiske-Subbarow method (cited by Hawk, Oser and Sommerson, '48). Calcium determinations were made by precipitating as the oxalate at pH 4.0 and titrating with 0.01N $KMNO_4$. Bisulfite-binding substances were determined by the method of Shils et al. ('41) and the Benedict-Francke method cited by Hawk et al. ('48) was used for uric acid determinations. Heart and abdominal muscle samples were dried at 100°C., extracted in a Goldfish extractor with ether and dry ashed at 550°C.

RESULTS AND DISCUSSION

In a preliminary experiment determinations were made of the creatine excretion levels of cotton rats fed the basal ration (0.01% Mg) with vitamin E (0.05%), manganese (0.03%) or methionine (0.2%) supplements, and a weekly supplement of β-carotene in olive oil in place of haliver oil, or the basal ration containing 1% salts IV (0.01% Mg) with 1.4% $CaCO_3$ added (low minerals). Two litters of 6 animals each were used. The creatine excretion data in figure 1 showed that all animals, with the exception of one in the low minerals group and another in the manganese-supplemented group, excreted increasing amounts of creatine starting about the 14th day. The fact that the excretion level of these two animals continued to be 1.0 mg/24 hours or less for the entire period suggested that the creatine excretion level of cotton rats should normally be low. The weight gains and heart ash data which are given in table 1 showed that all animals of litter 347 were more severely affected than those of litter 324 and demon-

strated the necessity of having litter-mate controls in these studies.

In the previous survey report (Constant and Phillips, '52) it was found that vitamin E had no beneficial effect on growth or the ash content of the heart, a concurrent manganese deficiency increased the severity of the disease, and a reduction of minerals other than calcium resulted in better growth

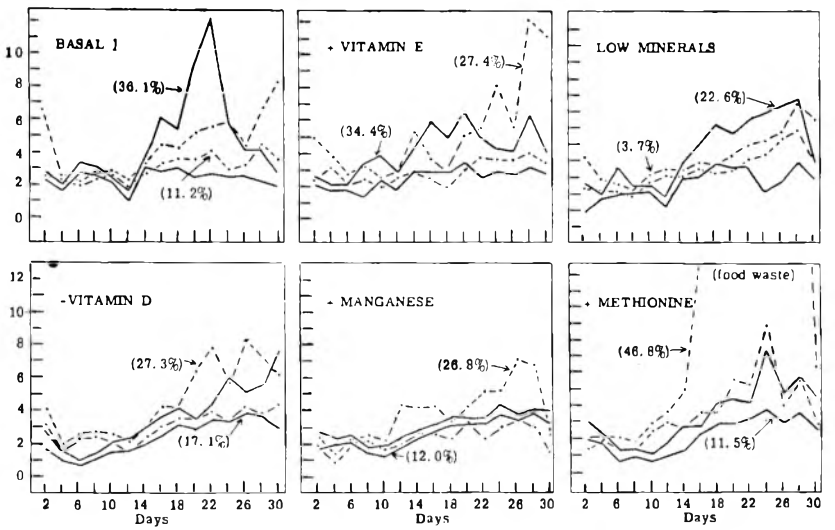

Fig. 1 Daily excretion of creatine and creatinine — mg/24 hr.*

* Data from two 24-hour collections are averaged for each point. Each pair of broken or solid lines designates excretion data on individual male and female rats which are further identified in table 1. Total creatinine values are graphed on upper curve, "free" creatinine values on lower curve; thus, distance between points on a given pair of solid or broken curves equals creatine, expressed as creatinine. Data in parentheses equal % ash content of heart.

and a reduced ash content of the heart. Although vitamin E reduced the creatinuria of guinea pigs deficient in the anti-stiffness factor (van Wagtendonk et al., '44), it apparently had no effect on the creatine excretion levels of cotton rats. The present experiment indicated that the calcinosis was not due to excessive intakes of vitamin D, or to an increased requirement of the cotton rat for manganese or methionine.

In the second experiment the effect of increasing levels of magnesium on the severity of the disease and the excretion of chloride, phosphorus and creatine was studied. The basal ration which contained 0.02% Mg was supplemented with $MgSO_4 \cdot H_2O$ to provide an additional 0.04% and 0.08% Mg.

TABLE 1

The variation in the susceptibility of litters to the disease

RATION	WEIGHT GAIN		HEART ASH	
	Litter no. 324	Litter no. 347	Litter no. 324	Litter no. 347
	gm	*gm*	%	%
Basal	34 ♂	27 ♀	11.2	36.1
+ vitamin E	27 ♂	27 ♀	27.4	34.4
avitaminosis D	21 ♀	43 ♂	17.1	27.3
+ manganese	35 ♀	29 ♂	12.0	26.8
+ methionine	29 ♀	23 ♂	11.5	46.8
range	(23–43) 5 ♂	(21–35) 5 ♀	(11–27)	(27–47)
Low minerals	59 ♂	47 ♀	3.7	22.6

TABLE 2

Composition of diets which were fed to weanling white rats

LOT	1	2	3	4	5	6	7	8
Mg (mg %)	40	10	10	10	10	10	10	10
Ca pantothenate (mg/kg)	30	30	10	10	30	10	30	10
Weekly haliver oil supplement [1]	*	*	*	**	**	**	**	**
Other changes					(1)	(2)	(3)	(4)

(1) Biotin, 10 mg/kg; (2) cortisone, 2 mg/10 gm; (3) No KI; (4) iodinated casein, 0.05%.

[1] All animals were given two drops of haliver oil every 8th day starting * immediately or ** on the 24th day.

The 4th rat of each of the 4 litters used in this experiment was fed cortisone in the ration at an initial level of 3.0 mg/10 gm of ration. The level was reduced to 1.5 mg on the 14th day because of the very poor growth of the animals, but they still failed to grow. The data in figure 2 show that food consumption and growth increased with increasing levels of

Mg in the ration and decreased in the cortisone-supplemented group.

Although there was a marked difference in the chloride excretion per 48 hours, the data in figure 2 show that, on the basis of food consumed, there was no difference between the

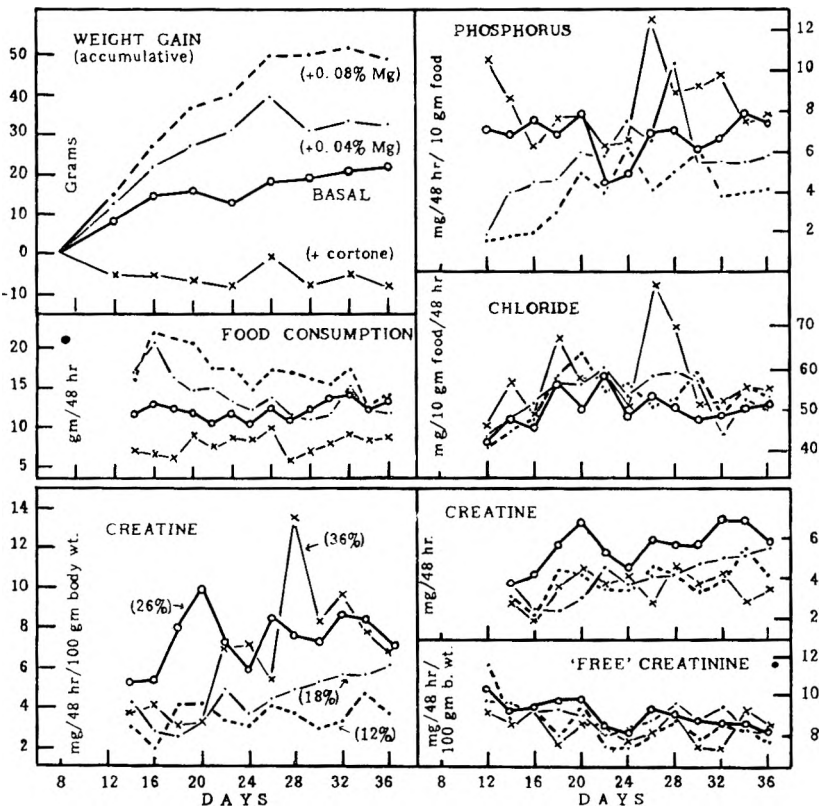

Fig. 2 The effect of increasing levels of magnesium and of cortisone on the disease.

various groups. On the other hand, the phosphorus excretion data computed on the basis of food consumed showed that the animals were divided into two groups, low Mg and high Mg. This finding has been repeated in each of the experiments where a high Mg ration was used as a positive control. The basal group excreted the greater amount of creatine

throughout the experiment. Although cortisone increased catabolism (Ingle, '50), and these animals showed considerably poorer growth and more extensive calcification than the basal group, their creatine excretion was not greater than that of the basal group. Beard ('46) reported that creatine excretion increased with increased protein intake. In an attempt to assess this influence on creatine excretion, the data were computed on the basis of 100 gm of body weight. These data (fig. 2) showed a direct correlation with the terminal severity of the disease, as indicated by the ash content of the hearts of these animals. In addition to lesions of the heart and skeletal muscle, the animals fed cortisone showed large (1-cm) areas of calcification of the subcutaneous fat in the thoracic region.

The previously reported studies had shown that when dextrin replaced the dietary sucrose, the animals showed normal growth despite the low level of magnesium in the diet. In the third experiment, 4 litters of 4 animals each were fed the basal ration with sucrose, or with dextrin, or with dextrin and 0.08% Mg, or with sucrose and 0.05% $ZnCl_2$. The weight gains, the ash, and the calcium content of cardiac and skeletal muscle are given in figure 3. These data confirm the previous findings; namely, that the substitution of dextrin for sucrose resulted in normal growth despite the low magnesium content of the diet (Constant and Phillips, '52). The heart ash content was reduced from an average of 27% to 13%; the calcium content of heart and skeletal muscle was reduced from 4.2% to 2.1% and 3.8% to 1.7%, respectively. The ash content of the abdominal muscle was not reduced. The presence of additional Mg (0.08%) in the diet resulted in a further decrease in the ash content of the heart to 7.2% and the calcium content to 1.0%. The ash content of the abdominal muscle was reduced from 10% to 4% and the calcium content to 0.1%.

The food consumption data (fig. 3) show that the presence of dextrin or dextrin and Mg prevented the decline in appetite which occurred when the low magnesium-sucrose diet was fed. The presence of Mg in the diet resulted in a consistently

greater excretion of calcium, whereas there was no difference in the calcium excretion of animals fed the low magnesium-sucrose or dextrin diets. The phosphorus excretion of animals fed the basal diet with dextrin and Mg was consistently lower than that of the animals fed the low Mg diets. The presence of dextrin tended to decrease the phosphorus excretion. From the 12th to the 20th day the amounts excreted

RATION	Graph Symbol	No. of rats	Weight Init.	Weight Final	HEART % Ash[1] Ave. ± S.D.	HEART Calcium[2] Ave. ± S.D.	ABDOMINAL MUSCLE % Ash[1] Ave. ± S.D.	ABDOMINAL MUSCLE Calcium[2] Ave. ± S.D.
			gm	gm				
BASAL		4	48	53	26.7 ± 8.4[3]	4.2 ± 1.0	9.9 ± 1.3	3.8 ± 2.1
" with dextrin		4	49	77	13.2 ± 8.7	2.1 ± 1.4	10.1 ± 3.2	1.7 ± 0.7
" " " + Mg		4	44	73	7.2 ± 3.1	1.0 ± 0.8	4.3 ± 1.4	0.1 ± <0.1
" + Zn		4	45	57	23.4 ± 1.1	5.8 ± 0.8	13.2 ± 4.4	2.1 ± 0.4

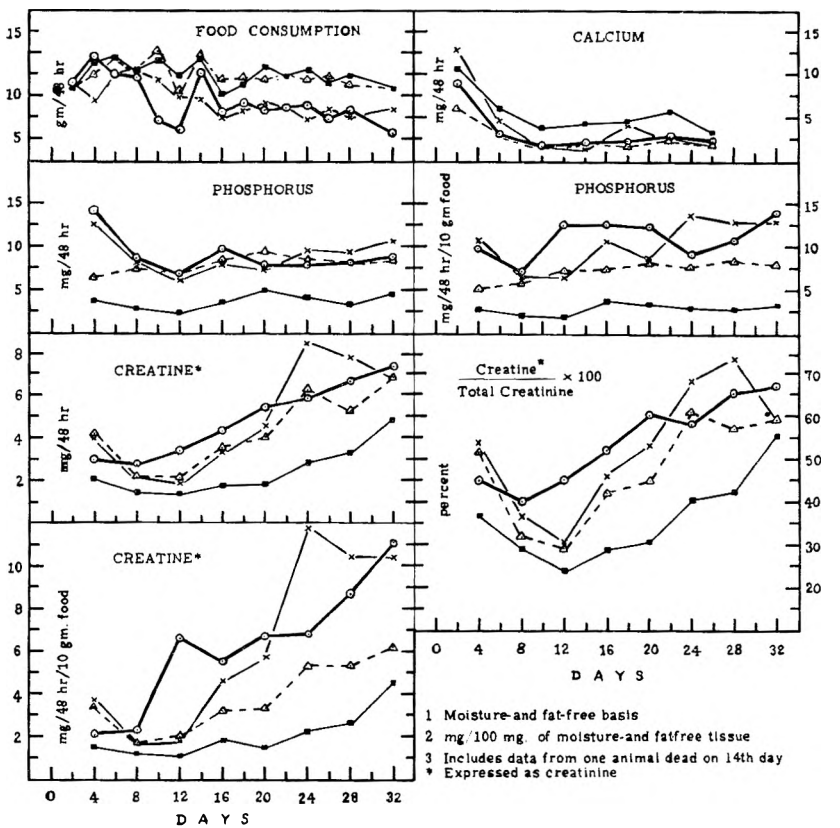

Fig. 3 The effect of dextrin or magnesium on the disease.

by the low Mg-dextrin group were less than the range of amounts excreted by the animals fed the low Mg-sucrose ration. The creatine excretion data in figure 3 show that there was an increase in creatine excretion with time in all groups. However, the excretion level of the basal sucrose group was at all times greater than that of the group fed the basal ration with dextrin and Mg. The excretion values of the low Mg-dextrin group tended to be intermediate. The differences between the groups were increased when the effect of protein was taken into account by computing the data on the basis of the amount of food consumed. Calculations on the percentage of creatine excreted in relation to total creatinine showed high values for the basal ration, low values for the basal with dextrin and Mg, and intermediate values for the basal with dextrin.

In the 4th experiment animals were fed the basal diet supplemented with biotin (5 mg/kg) or aureomycin. The initial level of aureomycin was 250 mg/kg, which was reduced to 50 mg/kg on the 4th day and to 0 mg/kg on the 12th day because of severe constipation apparently caused by the aureomycin. These animals did not defecate until after they had been on the basal ration for approximately 4 days. The feces were slate grey and were decreased in quantity for almost the remainder of the experiment. Aureomycin (50 mg/kg) was added to the diet again on the 18th day. The basal diet was fed to a negative control group and the stock diet to the positive control group. At the end of the experiment, animals which had been fed the stock diet were switched to the basal diet and kept on experiment an additional two weeks to compare the susceptibility of animals two months old with that of weanlings.

Analyses of cardiac and abdominal muscle showed (fig. 4) that supplements of biotin or aureomycin did not prevent or reduce the calcinosis. Although one group had been fed the stock diet during their period of rapid growth before the basal diet was fed, these animals showed an average of 13% ash in the heart, which is approximately three times that of

RATION	Graph Symbol	No. of rats	WEIGHT Initial	WEIGHT Final	HEART % Ash[1] Ave. ± S.D.	HEART Calcium[2] Ave. ± S.D.	ABDOMINAL MUSCLE % Ash[1] Ave. ± S.D.	ABDOMINAL MUSCLE Calcium[2] Ave. ± S.D.
			gm	gm				
BASAL	—○—	4	45	78	26.6 ± 4.5	4.3 ± 0.2	12.1 ± 1.9	1.9 ± 0.5
" + Biotin	—△—	4	45	81	31.5 ± 8.5	4.3 ± 0.9	11.3 ± 2.6	1.7 ± 0.9
" + Aureomycin	—×—	4	42	70	21.4 ± 6.2	4.2 ± 1.4	9.3 ± 1.0	1.0 ± 0.7
STOCK DIET, then BASAL	—□—	4	44	89 (85)	13.2 ± 2.8	1.5 ± 0.5	6.6 ± 1.1	0.5 ± 0.1

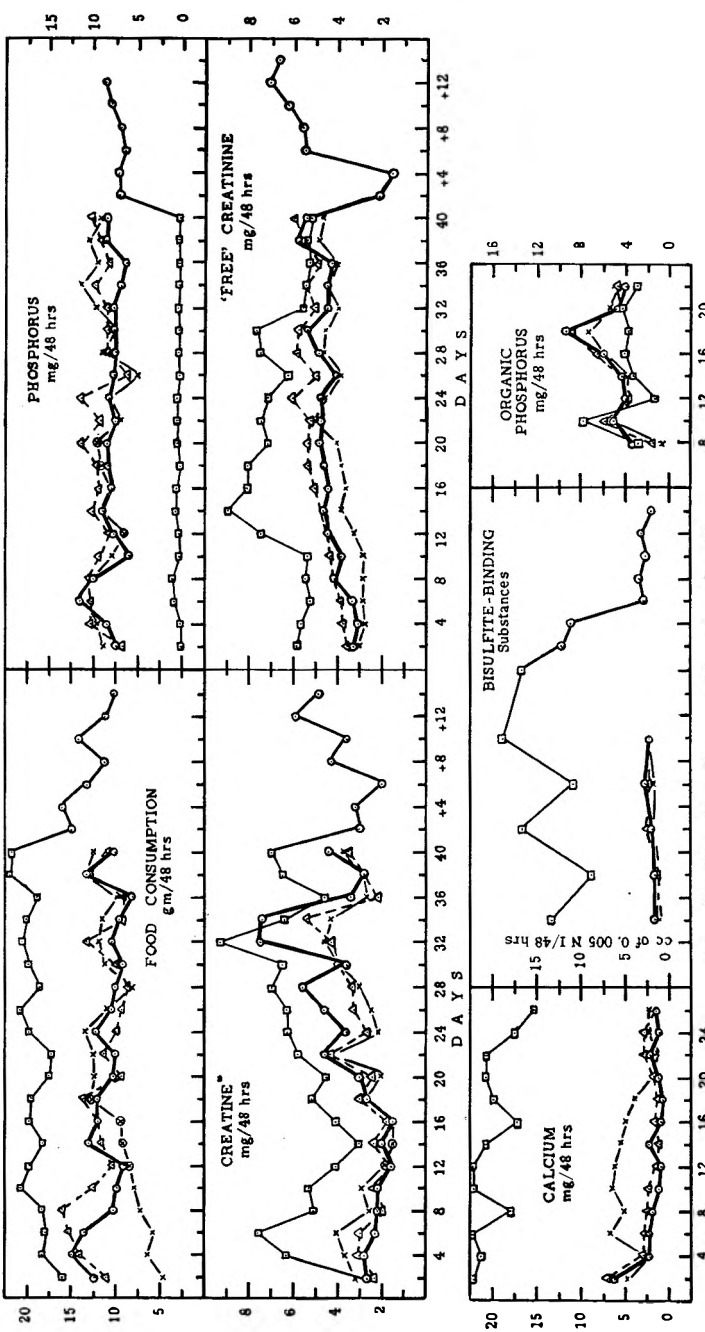

Fig. 4 The effect of biotin, aureomycin or age on the disease. Footnotes 1, 2 and * are the same as for figure 3.

animals maintained continuously on the stock diet (fig. 6). Despite the considerably greater food consumption of animals fed the stock diet and the higher level of phosphorus in that diet, the phosphorus excretion (fig. 4) was very low, averaging approximately 0.5 mg/48 hours for the entire period, compared to 5 to 10 mg for the groups of animals fed

RATION	Graph Symbol	No. of rats	WEIGHT Initial	WEIGHT Final	HEART % Ash[1]	HEART Calcium[2]	ABDOMINAL MUSCLE % Ash[1]	ABDOMINAL MUSCLE Calcium[2]
			gm	gm	Ave. ± S. D.	Ave. ± S. D.	Ave. ± S. D.	Ave. ± S. D.
BASAL[3]	-o-	3	201	186	6.6 ± 0.8	0.4 ± <0.1	5.0 ± 0.2	0.1 ± <0.1
" with dextrin + Mg	-Δ-	3	175	185	6.6 ± 2.4	0.2 ± <0.1	4.6 ± <0.1	0.1 ± <0.1
STOCK DIET (S.D.)	-x-	3	178	—	4.8 ± 0.1	0.2 ± <0.1	4.8 ± 0.2	0.1 ± <0.1

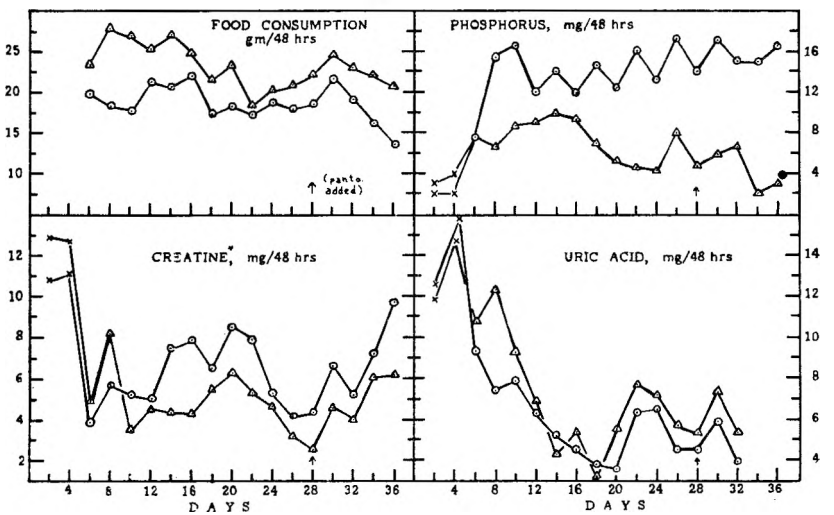

Fig. 5 The decreased susceptibility of adult animals to the disease. Footnotes 1, 2 and * are the same as for figure 3.

[3] Adult animals (6 months old) were fed pantothenate-deficient diets until the 28th day.

the low Mg basal. Immediately upon being switched to the basal diet, the phosphorus excretion rose to approximately 7 mg/48 hours. The creatine and "free" creatinine excretion of animals fed the stock diet was quite variable but in almost all instances was greater than that of animals fed the synthetic diets. The free creatinine excretion dropped sharply to approximately 2 mg/48 hours for 4 days after feeding of

the basal diet and then rose sharply to 6 or 7 mg. The creatine level also dropped to 2 to 3 mg for 6 days and then rose sharply but irregularly to 5 or 6 mg/48 hours. This rise was earlier and sharper than that shown by animals fed the basal diet at weaning age. The calcium excretion of animals fed the stock diet was several times that of animals fed the synthetic diets.

The decrease in appetite shown by animals fed the low magnesium diets and the interrelationship of magnesium to thiamine-containing enzymes suggested the possibility of a thiamine deficiency. Shils et al. ('41) showed that the excretion of bisulfite-binding substances was greatly increased in thiamine-deficient white rats. However, it was found that the excretion of these substances by animals fed the stock diet was considerably greater than that of animals fed synthetic diets (fig. 4) and dropped gradually to a low level after the animals were switched from the stock diet to the basal. Preliminary determinations of organic phosphorus excretion indicated that the excretion level of this substance may also be less when diets are fed which do not precipitate the calcinosis. These findings were checked further in the 6th experiment.

Since animals which were two months old were as susceptible to the development of the disease as weanlings, adults 6 months old were used in the 5th experiment. These animals were fed the basal diet (0.02% Mg) or the basal diet with dextrin and Mg (total of 0.1%). Both diets were deficient in calcium pantothenate (see discussion on enzyme studies). They were fed the stock diet for 4 days prior to being fed the synthetic diet. All animals fed the basal diet had a few white linear streaks observable grossly in the heart muscle. One of the animals fed the basal with dextrin and Mg had an atypical lesion which resembled a scar, and the ash content of the heart of this animal was 10% compared to 4.8 and 5.1% for the other two animals of this group and 5.7 to 7.6% for the basal group. The calcium content was 0.5% compared to 0.1% for the other two animals and 0.4 to 0.5% for the basal

group. In comparison to animals three weeks or two months old, adults were much less susceptible to the development of the calcinosis. There was no increase in the ash or calcium content of the abdominal muscle (fig. 5).

The food consumption of adults fed the low magnesium basal was less than that of adults fed the basal with magnesium. The excretion data given in figure 5 show that phosphorus excretion rose abruptly when the synthetic diet was fed and the same increased excretion by the low Mg group was observed as in the case of weanlings. There was a definite tendency for the low Mg group to excrete greater amounts of creatine than the high Mg group, although this difference was not as pronounced as in weanlings. The uric acid excretion of the animals was considerably greater when they were fed the stock diet than when they were fed the synthetic diets.

In the last experiment in which cotton rats were used the animals were fed the basal diet supplemented with vitamin A [3] (10,000 U.S.P. units/kilogram) or calcium pantothenate (total of 70 mg/kg). The basal diet plus Mg (total of 0.1%) or with dextrin and Mg (total of 0.1%) was used as the positive control. In a preliminary experiment, 8 pairs of weanling male white rats were fed the basal diet containing one-half as much magnesium (0.01%) or otherwise modified as shown in table 2. The cotton rats were fed the stock diet for 8 days and the white rats for 4 days prior to feeding the experimental rations.

The data on the ash and calcium content of cardiac and skeletal muscle which are given in figure 6 for each group of cotton rats showed that the vitamin A or calcium pantothenate supplements had no beneficial effect on growth or deposition of minerals in muscle tissue. Supplementation of the diet with Mg improved growth and reduced the ash and calcium content of the tissues to the range of values obtained for animals maintained on the stock diet. Although the pairs of white rats fed cortisone or iodinated casein grew very poorly,

[3] Nopcay "20" Type IV, 10,000 U. S. P. units/gram; Nopco Chemical Co., Harrison, N. J.

the ash and calcium contents of cardiac and skeletal muscle were not increased thereby. All of the cotton rats fed the high magnesium diet with sucrose had a few short white linear streaks observable grossly on the heart, whereas none was visible on the hearts of animals fed the high Mg diet

RATION	No. of rats	Graph Symbol	WEIGHT Initial	WEIGHT Final	HEART % Ash[1]	HEART Calcium[2]	ABDOMINAL MUSCLE % Ash[1]	ABDOMINAL MUSCLE Calcium[2]
			gm	gm	Ave. ± S.D.	Ave. ± S.D.	Ave ± S.D.	Ave. ± S.D
BASAL	3		55	75	31.3 ± 3.6	4.2 ± 0.6	12.7 ± 5.8	3.7 ± 2.3
" + Vitamin A	3		50	68	31.3 ± 5.3	7.4 ± 0.6	11.7 ± 5.4	3.6 ± 2.0
" + Calcium pantothenate	3		48	74	31.8 ± 3.7	7.2 ± 1.1	9.9 ± 2.8	2.2 ± 1.2
" + Magnesium	3		48	106	5.8 ± 0.5	0.2 ±<0.1	4.8 ± 0.3	1.2 ± 0.1
" with dextrin + Magnesium	3		43	103	5.1 ± 0.6	0.2 ±<0.1	4.6 ± 0.2	0.2 ±<0.1
STOCK DIET (S.D.)	7		(3 mos.	old)	3.5 ± 1.4	0.1 ±<0.1	5.8 ± 1.5	0.4 ± 0.6
WHITE RATS	16		37	127				
(cortone)	–		–	64	4.1 ± 0.8	0.1 ±<0.1	6.6 ± 3.3	0.1 ±<0.1
(iodinated casein)	–		–	75				

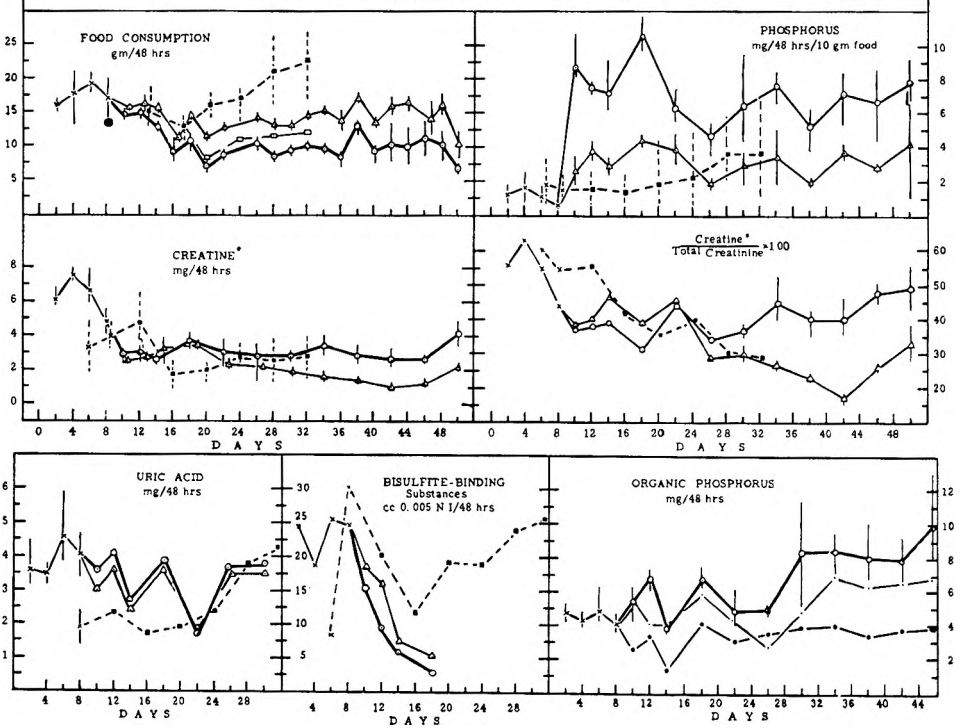

Fig. 6 Comparison of the effect of low Mg diets on the ash content of tissues and the urinary excretions of white rats and cotton rats.

Footnotes 1, 2 and * are as for figure 3.

The values for the three groups of cotton rats fed low Mg diets, two groups fed high Mg diets and 8 pairs of white rats are averaged and plotted. The maximum and minimum average of the subgroups are given on the vertical lines.

with dextrin. The presence of these lesions was not reflected by an increase in the ash or calcium content of the heart and, as noted previously (Constant et al., '52), the early lesions may not be accompanied by calcium deposition.

The food consumption data in figure 5 again showed the effect of magnesium in maintaining the appetite. The amount of food consumed during the first week of feeding the synthetic diets to white rats and cotton rats was similar. Thereafter, food consumption by the white rats increased until it was approximately one and one-half to two times that of the cotton rats. Although 14 of the 16 white rats were fed diets containing one-half as much magnesium as was contained in the low Mg diets fed to the cotton rats, there was no immediate rise in phosphorus excretion upon switching from the stock diet to the synthetic diet. The white rats fed iodinated casein excreted the greater amounts of phosphorus in the last 4 collection periods. Otherwise, all pairs of animals excreted less than 4 mg/10 gm food. The vitamin supplements to the diets of the cotton rats modified the excretion levels of phosphorus by the low Mg groups, but the levels of excretion were not reduced to the level shown by the high Mg groups.

As in previous experiments, cotton rats fed the low Mg basal ration excreted greater amounts of creatine than those fed the Mg-supplemented ration. The excretion levels of the white rats were low for two of the early collections but rose when the food consumption increased. If the data were calculated to take into account the difference in protein consumption, the amounts of creatine excreted by white rats would be less than the creatine excretion by cotton rats fed the Mg-supplemented ration. The low Mg cotton rat groups excreted the highest percentage of creatine. The white rats showed a gradual decline in the percentage of creatine excreted, and when the experiment was terminated the values were similar to those of the cotton rats fed the Mg-supplemented diets.

The excretion of uric acid by white rats was less than that of cotton rats when the stock diet was fed and in the early

collection periods when synthetic diets were fed, but it increased when food consumption increased. There was no difference in the uric acid excretion of cotton rats fed the low or high Mg diets. The drop in the excretion level of bisulfite-binding substances which was noted previously (fig. 4) was again observed. The data obtained with white rats indicated that the excretion level was influenced by the food consumption level. However, the low level excreted by cotton rats indicated that according to this criterion the cotton rats were not suffering from thiamine deficiency. The previous indication that the organic phosphorus excretion may also be affected by diet or the disease was checked in this experiment. The data (fig. 6) showed that the organic phosphorus excretion of animals fed the basal diet with dextrin and Mg was less than that of animals fed the basal diet with sucrose and Mg. Although there were no overlapping values for the last 4 collections of samples, a definite conclusion as to the effect of dextrin on the excretion of this substance cannot be made until the experiment is repeated.

In view of the fact that an increase in the protein content of the diet showed some beneficial effect on this disease (Constant and Phillips, '52) and that Williams and Elvehjem ('49) have shown that xanthine oxidase activities may be used as an assay for protein quality, studies were made of the xanthine oxidase activities of liver homogenates by the method of Axelrod and Elvehjem ('41). Control flasks containing homogenates of the livers of cotton rats fed the stock diet, or white rats two to three months old fed the stock diet, were used in each series of determinations. Duplicate flasks were used for endogenous respiration and xanthine oxidase activity determinations. Only the values for endogenous respiration are shown in figure 7, for in approximately 80 to 90% of the determinations there was a marked decrease in oxygen uptake when the xanthine substrate was added and the inhibition remained for the duration of the test (240 minutes).

The high endogenous respiration of homogenates of the livers of cotton rats compared to that of the livers of white rats of similar age is shown in figure 7. The maximum oxygen uptake by liver homogenates in the case of white rats was shown in the first 10 minutes, gradually declined and

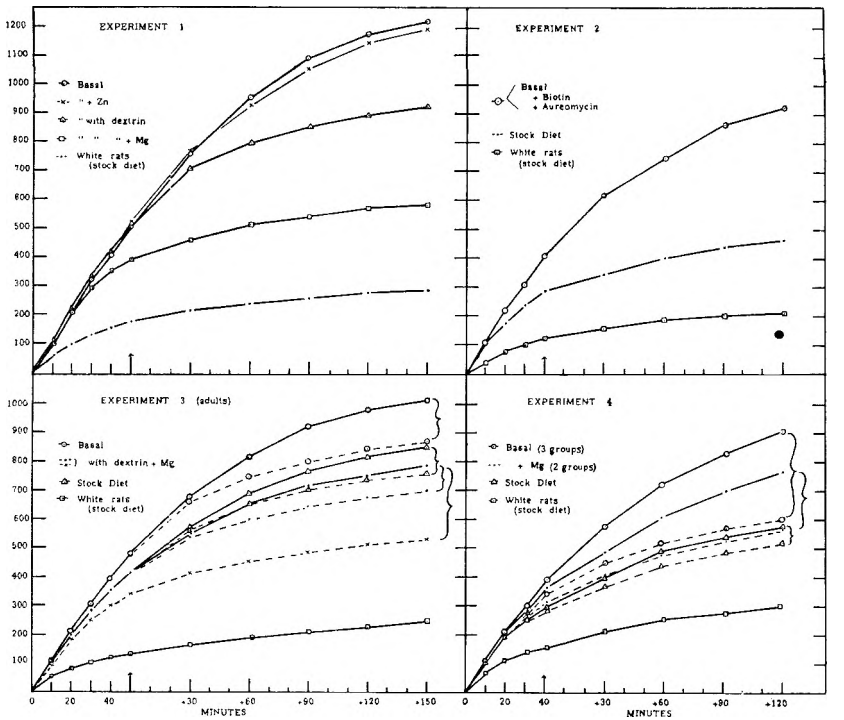

Fig. 7 The oxygen uptake of homogenates of the livers of cotton rats and white rats — μl O_2 per flask (284 mg of tissue).

Dotted lines represent values obtained when 100 μg of calcium pantothenate were added in vitro. Animals used in experiments 1, 2, 3 and 4 of these studies were those used in experiments 3, 4, 5 and 6 of the urinary excretion studies.

leveled off at approximately 40 to 50 minutes. The oxygen uptake of homogenates of livers from weanling and adult cotton rats was linear in all cases for the first 30 minutes and in the case of animals fed low Mg diets it continued at the same rate for 50 minutes. The data in figure 7 show that the oxygen uptake of homogenates of the livers of cotton

rats fed the stock diet or the basal diet supplemented with magnesium tapered off more rapidly than that of the livers of animals fed the low Mg diets.

In an attempt to modify the oxygen uptake, several substances were tested. Of these substances — vitamin C, glutathione, pyruvic acid, NaF, iodoacetic acid, magnesium, methionine, sodium citrate, folic acid or calcium pantothenate — only calcium pantothenate produced a significant and consistent change in the rate of oxygen uptake. When 100 μg of calcium pantothenate were added per flask with the xanthine, the oxygen uptake gradually decreased and was significantly less at the end of 60 minutes. If calcium pantothenate was added at zero time, the accumulative decrease was considerably greater but it still took approximately 40 to 50 mintues before the rate of uptake was significantly less than that of flasks containing a similar volume (0.1 ml) of water. This procedure, however, did not reduce the oxygen uptake to the level shown by homogenates of the livers of white rats. The addition of calcium pantothenate to the homogenates of livers of white rats fed the stock diets had no effect on the oxygen uptake. Dietary supplements of biotin, aureomycin, vitamin A or calcium pantothenate had no effect on the endogenous respiration of the liver homogenates.

In addition to the similarities between the calcification disease in guinea pigs and cotton rats (Constant et al., '52; Constant and Phillips, '52), several other similarities have been observed in the present studies. Guinea pigs fed diets deficient in the anti-stiffness factor showed high respiration of liver homogenates (van Wagtendonk and Wulzen, '50), similar to that of the deficient cotton rats. It is of interest to note the low xanthine oxidase activity of guinea pig livers (Richert and Westerfeld, '51) and the difficulty in demonstrating xanthine oxidase activity in homogenates of livers of cotton rats. Roine and Ettala ('52) have recently reported the toxicity of aureomycin (100 mg/kg) for guinea pigs, and in the present study a low tolerance for aureomycin was found with cotton rats. Generalized hair loss is a non-specific

symptom of ill cotton rats. Cotton rats fed the low Mg diets began losing excessive quantities of hair after the first week and those fed Mg-supplemented diets did not start shedding excessively until about the 4th week. Hair loss never reached the stage of alopecia. A more marked loss of hair by severely deficient guinea pigs was observed by van Wagtendonk and Wulzen ('50). An observation made with cotton rats which has not been reported for guinea pigs was a decrease in the color of the feces, which were brown at the beginning of the experiment and progressively faded to colorless in almost all animals fed the low Mg diets for three weeks or longer. The loss in color of the feces was considerably slower when high Mg diets were fed and often there was no appreciable change.

The actual amounts of creatine excretion varied somewhat from experiment to experiment but the increased excretion of creatine by animals fed low Mg diets (0.02% Mg) compared to animals fed diets containing 0.1% Mg was similar. Substituting β-carotene in olive oil for the haliver oil supplement had only a very temporary reducing effect on creatine excretion, and supplementing the diet with stabilized vitamin A had no effect on the calcinosis. The early decrease in appetite and poor growth suggested a possible increase in the requirement for B vitamins, but the addition of vitamin supplements to a low Mg-dextrin diet was previously found to have no alleviating effect on the disease (Constant and Phillips, '52) and in the present studies supplements of biotin or calcium pantothenate had no effect. Although aureomycin has been shown to have a sparing action on some B vitamins in sucrose-containing diets (Lih and Baumann, '51), supplementing the low magnesium diet with aureomycin was not beneficial. Many of the supplements modified the creatine excretion levels of cotton rats but none was as effective as Mg in reducing the creatine excretion.

White rats which consumed the same basal diet containing 0.01% Mg instead of 0.02% Mg did not show the immediate rise in phosphorus excretion which was found in each

experiment with cotton rats. Dogs (Kruse et al., '34) which were fed diets containing 1 to 2 mg % of Mg did not show this immediate phosphaturia. White rats required approximately 5 mg % of Mg when the diet contained 0.8% calcium for normal growth (Tufts and Greenberg, '38), whereas the low Mg basal ration which resulted in extensive calcification in cotton rats contained 20 mg % of Mg and 0.56% Ca. The generalized calcification which occurred when cotton rats were fed diets containing 20 mg % was not found when white rats were fed diets containing 2 mg % Mg (Lowenhaupt et al., '50). However, it has been found that calves on Mg-low diets showed calcification of arteries and frequently of skeletal muscle (Moore et al., '38) and that guinea pigs which developed lesions similar to those in cotton rats (Harris and Wulzen, '50) showed a marked improvement in growth when the dietary levels of K and Mg were raised to 1% and 0.3%, respectively (Roine et al., '49).

The increased creatine excretion and the immediate phosphaturia which occurred when cotton rats were fed diets containing 20 mg % of Mg, when compared to the effects of diets containing 0.1% Mg, suggest that the calcinosis syndrome was due to a magnesium deficiency. However, the alleviating effect of dextrin, protein or decreased dietary minerals other than calcium indicates that the intake of other nutrients may have been imbalanced or suboptimum. This suggestion is further borne out by the fact that lesions were observed grossly when a partially purified diet containing 0.1% Mg and sucrose was fed. To date animals fed a synthetic diet with dextrin and 0.1% Mg have not been kept on experiment longer than 50 days and it is not known whether lesions would eventually develop.

Although these studies have been of considerable value in obtaining information concerning the disease, histopathologic examination of the tissues must be used as the final criterion for determining its presence.

SUMMARY

Several experiments have been conducted to study the effect of diet and age on the susceptibility of cotton rats to development of the calcinosis disease.

Partially purified diets which were low in Mg (0.02%) precipitated and increased the severity of the disease compared to diets containing 0.1% Mg. An immediate phosphaturia occurred when cotton rats were fed the low Mg diets which was not shown by white rats fed diets containing 0.01% Mg or by cotton rats fed diets containing 0.1% Mg. The creatine excretion of cotton rats fed the low Mg diets also was increased, starting at approximately the second week.

Supplementation of the low Mg diets with biotin, aureomycin, vitamin A, calcium pantothenate, vitamin E, manganese, Zn or methionine or omission of the vitamin D supplement did not improve the growth of the cotton rats or reduce the deposition of minerals in cardiac and abdominal muscles. Some of these supplements reduced the phosphaturia and creatinuria, but the reduction was not as great as that due to a dietary supplement of Mg. The substitution of dextrin for sucrose retarded the development of the disease.

Young animals were more susceptible to the disease than adults.

Several similarities have been noted between the disease in guinea pigs and in cotton rats.

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STUDIES ON THE RESPONSE OF LIVER XANTHINE OXIDASE TO DIETARY PROTEIN IN WEANLING RATS [1]

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INTRODUCTION

Several studies have been carried out in this laboratory relating the xanthine oxidase activity in the liver of adult rats to the quality of dietary protein (Litwack et al., '52, '53c). The behavior of this enzyme toward fasting and protein and amino acid-deficient diets (Miller, '48; Seifter et al., '48; Litwack et al., '50; Williams and Elvehjem, '49, '50; Williams, Denton and Elvehjem, '49) has been extensively studied, as have the effects of non-specific ammonium nitrogen sources on the enzyme activity (Litwack, Williams and Elvehjem, '53b).

In some of our previous work using adult animals (Litwack et al., '52, '53c) we have described a method for evaluating the quality of dietary protein, using the response in liver xanthine oxidase activity as the criterion. We have also tested the quality of various protein preparations, using this method. After studying the deficient protein, gliadin, and supplementation of this protein with amino acids, it was concluded that the enzyme response was very sensitive to

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[2] Fellow of the Williams-Waterman Fund for the Combat of Dietary Diseases.

changes in the state of the protein with respect to its amino acid composition. The results from the enzymatic method correlated quite well with growth studies carried out at the same time.

Since our previous enzyme work had been carried out almost exclusively with adult animals, it seemed important to extend these studies to weanling rats in order to observe changes in the enzyme activity while the organism was in a generative state with regard to tissue protein. Further information might also be gained concerning the possible competition for dietary protein between enzyme synthesis and growth. In addition, observations have been made on the possible effect of vitamin B_{12} on the response of liver xanthine oxidase to various dietary proteins, since the results of Williams et al. ('53a, b) have indicated the enhancement by this vitamin of protein value for growth and enzyme activity. The possible effects of molybdate ion, indicated by De Renzo and his associates ('53a, b) as activating intestinal and liver xanthine oxidase *in vivo,* were also included in these studies.

The proteins employed were casein, treated [3] casein, lactalbumin, treated [4] lactalbumin, gliadin, whole blood powder, treated [5] whole blood powder and corn-soya mixture.

A correlation has also been made between measurements of xanthine oxidase activity using manometric (Axelrod and Elvehjem, '41) and colorimetric techniques (Litwack et al., '53a). These results are compared with the observations on growth.

EXPERIMENTAL

Weanling, male, albino rats weighing between 45 and 50 gm [6] were used in these studies. Eight to 16 rats were employed in each enzymatic determination and a total of 384 animals was used in all of the experiments reported. The

[3] See section headed "Experimental" for the treatment of the protein.

[4] See footnote 3.

[5] See footnote 3.

[6] Holtzman-Rolfsmeyer Rat Co.

dietary procedure was identical to that described earlier (Litwack et al., '53c) except that 5, 10, 15 and 20% levels of protein were used throughout. The animals were housed individually in screen-bottomed cages and received food and water ad libitum. The fat-soluble vitamins were administered weekly in the form of two drops of halibut liver oil given orally. Weight data were recorded each week. The experimental feeding period for enzyme and growth determinations was 21 days, whereas the experimental period used for these measurements with adult rats in previous experiments was 9 days. This time interval was necessarily longer for the weanling animals since the enzyme activity in the younger animal is very low at weaning and for the first few weeks thereafter.

Xanthine oxidase was determined in the livers of the test animals at the end of the 21-day feeding period as described by Axelrod and Elvehjem ('41) at 37°C. The enzyme activity was also determined colorimetrically in most of the same liver homogenates simultaneously, with the manometric assay according to the method of Litwack, Bothwell, Williams and Elvehjem ('53a).

In the course of investigating possible effects of vitamin B_{12}, we wished to remove or destroy any vitamin B_{12} in the proteins being tested. Therefore, wherever such treatment has been indicated, the protein preparations were treated in the following manner: an equal volume of 0.1 N NaOH was added to the protein preparation, and this mixture was stirred and then autoclaved for 10 minutes at 15 pounds' pressure. After cooling, an equivalent amount of 0.1 N HCl was added for neutralization. The resulting gelatinous mass was sectioned into small chunks and dried in a hot air oven at 55°C. This product was finally ground in a Wiley mill into a homogeneous preparation for incorporation into the experimental diets. Crystalline vitamin B_{12} was added to the vitamin mixture (Williams and Elvehjem, '50) wherever indicated so that 100 gm of ration contained 2 μg of the vitamin.

The corn-soya mixture was prepared using a 1:1 ratio of whole ground corn to soybean oil meal. The casein employed was a vitamin-test preparation.[7] Lactalbumin,[8] gliadin[9] and the defatted and desiccated whole blood powder[10] were obtained from commercial firms. All diets were prepared isonitrogenously to a casein standard.

In the experiments involving the effect of molybdate ion, sodium molybdate was added to the protein at a level of 0.5 mg %. This product was then assayed by the enzymatic method, as described, at 5, 10, 15 and 20% levels of protein in the diet. Thus in the ration containing 20% protein, the level of sodium molybdate was 1 mg per kilogram of ration.

RESULTS AND DISCUSSION

A summary of the data for several protein preparations is presented in table 1. It should be pointed out that the value for xanthine disappearance in the colorimetric method has been converted to oxygen consumption, on the basis of one molecule of oxygen's being required for the oxidation of one molecule of xanthine to allantoin.

In the casein experiments, enzyme constants of 3.5 and 3.4 (μl O_2 per hour per gram liver per per cent of dietary protein) were obtained using manometric and colorimetric methods, respectively. The weight response constant was one and six-tenths (change in weight per week per per cent of dietary protein). It should be noted that the absolute numerical values for the enzyme response constant and the weight response constant should not be identical, because of the difference in the units in which they are expressed. A change in one, however, should be reflected by a similar relative change in the other if there is a positive correlation between the two constants. Interestingly enough, the pretreatment of casein, originally designed to destroy any vitamin B_{12} in the prepa-

[7] From General Biochemicals, Inc., Chagrin Falls, Ohio.
[8] Supplied without charge by the Western Condensing Co.
[9] Obtained from the Huron Milling Co.
[10] A product of the Viobin Corporation.

TABLE 1

Response of liver xanthine oxidase and growth in weanling rats to various protein preparations

PROTEIN STUDIED	NO. OF RATS USED FOR THE XANTHINE OXIDASE DETERMINATIONS	XANTHINE OXIDASE RESPONSE CONSTANT [1] (μl O_2/hr./gm fresh liver/% dietary protein)			WEIGHT RESPONSE CONSTANT [1] (Change in gm/wk./% dietary protein)
		Manometric results	Colorimetric results	Ave.	
Casein	56	3.5	3.4	3.5	1.6
Treated casein [2]	56	7.9	8.6	8.3	1.9
Lactalbumin	24	2.9	4.9	3.9	1.8
Treated lactalbumin [2]	24	9.1	6.5	7.8	2.8
Gliadin	48	1.5	..	1.5	0.06
Blood protein	8	Animals died [3]			
Treated blood protein	8	3.6	..	3.6	0.2
Corn-soya	32	4.4	2.6	3.5	1.7

[1] See Litwack, Williams, Chen, and Elvehjem ('52) for theory and method of calculating the constants.

[2] See text for method of treating the proteins.

[3] Only three animals out of 8 originally placed on experiment survived after 21 days; two fed 5% protein and one fed 10% protein.

ration, increased the enzyme constant by more than 100%. This effect was manifested by a slight increase in the growth constant. The results with adult animals, using the same diets, showed the following enzyme response constants measured manometrically: casein, 3.8 (16 animals); treated casein, 7.1 (16 animals). If these figures are compared with those for the weanling rats in table 1, it can be seen that good agreement was obtained.

For the groups receiving untreated and treated lactalbumin, almost the same xanthine response constants were obtained as with untreated and treated casein, respectively. When lactalbumin was pretreated with dilute alkali and heat in a similar manner to the casein, again a more than two-fold increase in the enzyme response constant was observed. In this experiment the growth constant was significantly increased in the group receiving the treated protein preparation.

Experiments with gliadin produced results which gave a slightly better enzyme response constant in weanling rats than that previously reported for adult rats (Litwack et al., '52, '53c), but the growth values were in good agreement with those previously reported.

When the untreated, defatted and desiccated blood protein preparation was used for the enzyme experiments, three out of 8 animals died within three weeks, apparently from starvation, since the animals refused to consume the diet. When this preparation was pretreated with alkali, however, the animals survived and produced an enzyme constant which was nearly equivalent to that of the untreated casein protein. It should be noted, however, that the growth constant was very low. This discrepancy between the enzyme response and growth cannot be explained at present. Whatever the detrimental factor in the untreated blood protein may be, its effect seems to have been overcome somewhat by the mild alkali-heat treatment. The results of this treatment may involve partial hydrolysis of the proteins. It is also possible that the effect is to make the proteins more soluble so that better

utilization by the animals occurs. Studies on this phenomenon are being extended at the present time in this laboratory.

Experiments with corn-soya diets produced enzyme response constants in weanling rats which were equivalent to those obtained with the lactalbumin protein. The growth values were also comparable.

From a comparison of the xanthine oxidase response constants with the weight response constants in these experiments, it appears that, although a fairly good correlation was obtained in most instances, this positive correlation was by no means completely consistent. It is possible that liver xanthine oxidase and general body tissues as measured by growth, are not produced at the same rates in weanling rats. For example, a certain dietary protein may enable the young, growing rat to produce normal liver xanthine oxidase levels but still may not be adequate for maximum growth. Since xanthine oxidase itself is a body protein, its response to dietary proteins should be a fair measure of protein metabolism but not necessarily of complete growth, which is the result of the effects of many other internal and external factors besides dietary protein.

Since, in work reported by Williams and co-workers ('53a, b) vitamin B_{12} appeared to enhance protein utilization, we decided to investigate the possibility that this factor might affect our determination of protein quality. The results of these experiments are shown in table 2, where casein, treated casein, gliadin and corn-soya diets were employed with and without added vitamin B_{12}. In most cases the measurements were made using both manometric and colorimetric procedures. It will be noted that for casein, treated casein and gliadin the inclusion of vitamin B_{12} increased the xanthine oxidase response to some extent. The possible significance of this response is made more apparent when the corresponding manometric results and colorimetric results (plus and minus vitamin B_{12}) as well as the over-all average are compared. The results with the corn-soya ration indicated no effect of vitamin B_{12}, however. We have noted that some

TABLE 2

The effect of vitamin B_{12} upon the response of liver xanthine oxidase to various dietary proteins

PROTEIN	$-B_{12}$				$+B_{12}$			
	No. of rats	Manometric K	Colorimetric K [1]	Ave.	No. of rats	Manometric K	Colorimetric K [1]	Ave.
Casein	32	3.1	..	3.1	32	3.9	..	3.9
Treated casein	32	9.6	8.7	9.1	32	10.3	9.5	9.6
Gliadin	32	1.6	2.7 [2]	2.1	32	2.0	3.5 [2]	2.7
Corn-soya	24	4.5	3.3 [2]	3.9	24	3.7	2.6 [2]	3.1

[1] When the coefficient of correlation between the manometric and colorimetric results of this table combined with those shown in table 1 was calculated (rank — difference coefficient of correlation), a coefficient of + 0.78 was obtained, indicating good agreement between the two methods.

[2] Sixteen animals were used to obtain these values.

TABLE 3

The effect of sodium molybdate added to the test protein on the response of xanthine oxidase [1]

PROTEIN	— MOLYBDATE			+ MOLYBDATE		
	Manometric K	Colorimetric K	Ave.	Manometric K	Colorimetric K	Ave.
Lactalbumin	5.0	4.9	5.0	4.8	4.6	4.7

[1] Sixteen animals were used in this experiment. The level of sodium molybdate added was 0.5 mg Na_2MoO_4/100 gm protein.

groups of rats, depending on their previous history, quite easily develop a vitamin B_{12} deficiency, while other groups are refractory to the deficiency. The latter condition may have obtained with the corn-soya-fed groups of rats.

De Renzo and co-workers ('53a, b) have suggested the role of molybdate ion in the activation of intestinal as well as liver xanthine oxidase. The activation of the intestinal enzyme by some factor had been established by Westerfeld and Richert ('49, '51; Richert and Westerfeld, '51). Since the liver enzyme had also been activated by inclusion of the molybdate ion in the diet, as described by De Renzo et al. ('53b), it was decided that this factor should be tested for its influence on measurement of the xanthine oxidase response constant. Sodium molybdate was added to the protein preparation so that the concentration of the ion would always be proportional to the level of protein. The results of this experiment are summarized in table 3. The molybdate did not alter the enzyme constant obtained. It would seem that the limiting factor for the enzyme constant under our conditions is controlled to a much greater extent by the concentration of dietary protein than by other factors whose influence might be more significant in the intestinal system. It should also be mentioned that a similar experiment was carried out with casein as the protein, and while this experiment produced somewhat erratic results, still no positive effect of the molybdate was obtained.

SUMMARY

1. Earlier studies on the response of liver xanthine oxidase to various dietary proteins, using adult rats, have been extended to weanling rats in order to compare the growth response with the enzyme response in the same animals.

2. A method of treating proteins which involves the addition of dilute alkali and autoclaving has been described. This treatment, originally intended to destroy vitamin B_{12}, was found to enhance the xanthine oxidase response in rats fed the treated proteins. Growth response was also increased to some extent by the treatment.

3. Under the conditions of these experiments the addition of molybdate did not alter the xanthine oxidase response constants of the proteins tested.

4. A slight stimulatory effect of vitamin B_{12} on the xanthine oxidase response was observed for three of the proteins tested.

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EFFECT OF COOKING AND OF METHIONINE SUPPLEMENTATION ON THE GROWTH-PROMOTING PROPERTY OF COWPEA *(VIGNA SINENSIS)* PROTEIN [1, 2]

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Dried mature seeds of the cowpea, *Vigna sinensis,* sometimes called Southern peas, are a staple in the diet of the people in the southern part of the United States. The protein content of these seeds varies between about 19 to 30%, depending upon the variety and the location in which they are grown (Wade et al., '51). The nutritive value, as measured by the gain response of young rats, also varies but the factors associated with this variation have not been ascertained.

The work of Finks et al. ('22), Sherman ('41), Richardson ('48), and Borchers and Ackerson ('50) has shown that the protein of cowpeas does not support a normal rate of gain in body weight in young rats. Sherman ('41) reported that

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the biological value of cowpea protein was improved by heating, and Borchers and Ackerson ('50) found that the growth-promoting property of autoclaved Blackeye cowpeas was superior to that of the uncooked seed. Finks et al. ('22), however, did not find any improvement in quality of protein following the cooking of the Brabham or Groit varieties, and Richardson ('48) reported that the protein quality of Blackeye cowpeas was not improved by autoclaving.

Finks et al. ('22) did not obtain any increase in the growth rate of their rats when the raw cowpea diets were supplemented with cystine, although Sherman ('41) reported that cystine improved the biological value of cowpea protein. Borchers and Ackerson ('50) increased the growth rate of their rats on raw cowpea protein diets when methionine was added as a supplement. They noted, however, that the increase was greater with one sample of Blackeye cowpeas than with another sample of the same variety. All of the above-cited investigators, as well as Jaffé ('49), obtained good growth response when cooked cowpeas were supplemented with cystine (Finks et al., '22; Sherman, '41) or with methionine (Richardson, '48; Jaffé, '49; Borchers and Ackerson, '50). All stated that methionine (or cystine) is the chief limiting amino acid in cowpea protein.

Some of the apparent discrepancies among the results of these investigators may have been due to differences in experimental techniques. There are indications, however, that the quality of cowpea proteins differs between samples of the same variety as well as between varieties. Thus Borchers and Ackerson ('50) found a difference between two samples of the Blackeye variety and Richardson ('48) reported the quality of the protein in Jackson Purple Hull to be inferior to that in Long Pod Cream or Dwarf California Blackeye No. 5.

The present work was undertaken to compare the growth-promoting properties of the proteins of 9 samples of cowpeas, both in the raw state and after being cooked, and to ascertain the effect of methionine supplementation on the growth re-

TABLE 1

Crude protein content of cowpeas and mean gain responses of rats fed these cowpeas as the sole source of protein

(The cowpeas were fed raw, cooked, and raw supplemented with methionine)

VARIETY	CRUDE PROTEIN (N × 6.25)		MEAN ADJUSTED [1] GAINS IN 21 DAYS			DIFFERENCES BETWEEN GAINS		
	Air dry basis							
	Raw	Cooked	Raw	Cooked	Raw + methionine	Cooked −raw	Methionine-supplemented −raw	Methionine-supplemented −cooked
	%	%	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Alacrowder [3]	24.63	24.63	30.0	40.7	51.8	10.7 * [2]	21.8 **	11.1 **
Long Pod Cream [3]	24.19	25.50	29.7	30.0	49.7	0.3	20.0 **	19.7 **
Chinese Red [3]	21.63	23.75	28.0	38.9	52.1	10.9 **	24.1 **	13.2 **
Bunch Purple Hull [3]	25.31	26.94	22.2	47.0	55.2	24.8 **	33.0 **	8.2 *
Alabunch [3]	22.24	23.81	31.4	40.6	53.6	9.2 *	22.2 **	13.0 **
Alabunch [4]	25.13	26.25	21.9	29.0		7.1		
Sugar Crowder [4]	23.81	24.69	23.4	30.1	52.8	6.7	29.4 **	22.7 **
Alalong [4]	24.63	26.00	24.5	44.9		20.4 **		
Groit [4]	25.06	27.13	27.9	37.3		9.4 *		

[1] Mean gains of 6 rats in a 21-day feeding period. Mean gains have been adjusted for group (block) effects (Cochran and Cox, '50).

[2] * = significant at the 5% level.

** = significant at the 1% level.

L.S.D. between any two adjusted means for P = 0.05 is 8.0; for P = 0.01 is 10.9 gm.

[3] Grown in Texas in 1947 as described by Wade et al. ('51).

[4] Grown in Oklahoma in 1947 as described by Wade et al. ('51).

sponse of rats subsisting on diets containing raw cowpeas as the source of protein.

EXPERIMENTAL

Cowpea samples

The mature dried seeds of the first 5 varieties listed in table 1 were received from the Department of Horticulture of the Texas Agricultural Experiment Station. The last 4 varieties in table 1 were from the Department of Horticulture of the Oklahoma Agricultural Experiment Station. All samples were grown in 1947 as described by Wade et al. ('51). They were sent to the North Carolina laboratory soon after harvest, where the feeding trials were conducted early in 1948.

A part of each sample was ground to pass a 10-mesh screen and used in the raw cowpea diets. Another part of the whole raw seed was covered with about three times its weight of water and allowed to stand overnight. Next day the swollen peas were heated for two hours in a covered double boiler. The cooked peas were spread out in thin layers and dried at 60°C. in a forced air oven, after which they were ground.

Diets

Diets containing 12% crude cowpea protein (N × 6.25) were compounded for each of the 9 samples of raw and the 9 samples of cooked cowpeas. All diets contained, in addition to the cowpeas, 9% hydrogenated vegetable fat, 1% cod liver oil, 1% $CaCO_3$, 0.5% NaCl and sufficient cornstarch to make up to 100%. Each 100 gm of diet was supplemented with 200 mg choline chloride, 40 mg *p*-aminobenzoic acid, 1 mg nicotinic acid, and 0.4 mg each of riboflavin, thiamine hydrochloride, pyridoxine hydrochloride and calcium pantothenate. In addition to these 18 diets, 6 others were used in which 0.3% methionine was substituted for an equal amount of starch in the raw cowpea diets.

Feeding

Because comparisons among 24 diets were involved, it seemed advisable to use an incomplete block design in which the rats in the blocks could be chosen for greater uniformity in initial weight and rate of gain in the preliminary period than would have been possible in a complete replication. A 5×5 balanced lattice, described by Cochran and Cox ('50) as plan 10.3, was adopted after adding a 25th diet, the nature of which is not pertinent to the present discussion. Under this plan the rats were assembled into groups (blocks) of 5 animals each. The animals in each group were chosen so as to be as uniform as possible in respect to sex, body weight, and rate of gain, as determined in a preliminary observation period of about one week.

Within the restrictions of the design the experimental feeding was randomized with respect to the diets, the groups within replications, and the rats within groups. Since Sherwood and Weldon ('53) found that ad libitum feeding was as sensitive as the three other procedures they used for detecting differences in gains in body weight, the individually caged rats were fed ad libitum for a 21-day experimental period.

RESULTS AND DISCUSSION

Although feed intake is not considered in the ensuing discussion of the results, the efficiencies of feed utilization have been computed for each rat used in this investigation and averaged for each of the experimental diets. The mean efficiencies of the rats on the raw cowpea diets varied from 0.125 to 0.212 gm gain per gram feed eaten. Those subsisting on the raw cowpea diets supplemented with methionine had efficiencies ranging between 0.276 and 0.326, and the efficiencies of the rats on the cooked cowpea diets varied from 0.158 to 0.251 gm gain per gram feed consumed.

Hegsted and Worcester ('47) have shown that comparisons between proteins may be made as effectively by the use of gain response as of protein efficiencies. Comparisons based

on gains in body weight, however, are over-all contrasts which include all factors affecting the gain response. In a single experiment in which the protein levels are held constant in otherwise comparable diets, the effects of extraneous factors are kept to a minimum and the gain responses measure primarily the adequacy of the protein for promoting growth, after the maintenance requirements are satisfied.

Comparison of raw samples

In general the differences among the responses to the various varieties of raw cowpeas (table 1) were less than the difference, 8.0 gm, required for significance at the 5% point. Hence, there is no clearcut evidence of a difference in the growth-promoting properties of the proteins of these samples of cowpeas.

The extremes in the responses, 21.9 and 31.4 gm, were from the two samples of the Alabunch variety which were grown in different locations. Furthermore, three of the 4 samples from Oklahoma produced gain responses of 24.5 gm or less, whereas 4 of the 5 Texas-grown samples stimulated gains of 28.0 gm or more. These observations suggest that the locations in which the cowpeas were grown were as important as variety in determining the quality of the protein. The evidence for a location effect is not conclusive, since the apparent superiority of one location over the other may have been due to the particular choice of varieties that were grown in each location or to the experimental error involved in testing the growth-promoting properties of the proteins.

Effect of cooking

Cooking over boiling water had practically no effect on the growth-promoting property of the protein in the sample of Long Pod Cream cowpeas, whereas the mean gain response of the rats on cooked Bunch Purple Hull cowpeas was approximately twice that of the rats consuming this variety in the raw state. In the other samples the improvement due to

cooking was intermediate between these two extremes. The growth-promoting effect of the protein in 6 of the 9 samples was significantly enhanced by cooking (table 1). The effect of cooking apparently was independent of the location in which the cowpeas were grown, and of the growth-promoting value of the raw protein. The findings of Richardson ('48) and of Borchers and Ackerson ('50), as well as the results reported here, indicate that the growth-promoting property of cowpea protein varies from sample to sample. Apparently the nutritive value of the protein is associated with the environmental conditions under which the plants are grown, and with variety.

Finks et al. ('22) attributed the increase in the nutritive value of cowpea protein following cooking to an increase in digestibility. Neither Fraps ('45) nor Jaffé ('50) found a significant increase in the digestibility of the protein of cooked cowpeas over that of the raw seed. Possibly the same process occurs in cowpeas as was found by Melnick, Oser and Weiss ('46) for soybean meal. Their results indicated that during digestion the methionine of the soybean meal was released earlier from the heat-processed meal than from the raw meal. Eventually the same amount of methionine was released and absorbed from either product but, in the case of the raw meal, absorption occurred so late that this amino acid was not efficiently utilized for the synthesis of body protein.

Methionine supplementation

The 6 methionine-supplemented diets stimulated significantly greater gain responses than the unsupplemented diets containing either raw or cooked cowpeas (table 1). The responses from the methionine-supplemented diets were not significantly different from each other. Thus methionine tended to equalize the growth-promoting value of the cowpea proteins regardless of their diversity in the raw or cooked states.

Methionine may not always equalize the quality of the protein, since Richardson ('48) found that a sample of Jackson

Purple Hull produced less gain response than either Dwarf California Blackeye No. 5 or Long Pod Cream after all three varieties had been autoclaved and supplemented with methionine.

Judging from the results reported in table 1 and those of others mentioned above, a lack of available methionine is the chief factor that limits the growth of young rats fed cowpeas as the sole source of their protein.

Experimental design

Although incomplete block designs have been widely used in agronomic research, their use in animal feeding experiments is rare. *A priori* these designs would be expected to be advantageous in reducing the experimental error whenever the animals can be assembled into groups (blocks) of individuals that are more homogeneous than is possible in a complete replication. No record of the employment of a balanced lattice for rat feeding has come to the authors' attention, although Cochran and Cox ('50) used the results of a nutrition experiment with swine as an example of the application of this design.

The data from the present study, when analyzed as a randomized block, yielded an error variance of 51.77. When analyzed as a balanced lattice, the effective error variance was 48.55, a reduction of about 7%. The use of the balanced lattice design in other unpublished rat-feeding experiments in this laboratory has resulted in gains in precision over the randomized block design in the order of 5 to 10%. In agronomic varietal tests, where the balanced lattice has been most widely used, the average gain in precision is in the order of 25% (Cochran and Cox, '50).

The relatively small gain in precision achieved by the use of the balanced lattice in the rat feeding experiment may have been due to the culling practiced before the rats were placed on experiment. Approximately one-third of the available weanling rats were discarded before the start of the feeding

trial because, in the judgment of the experimenters, they were unsuitable for experimental use. This culling may have reduced the variability of the remaining rats to such an extent that grouping on the basis of body weight and rate of gain in the preliminary period had little effect on the experimental error. There is the possibility, however, that the criteria used in grouping were not highly correlated with the variability in gain response. If this were true, the criteria were irrelevant and their use would not aid materially in reducing the experimental error.

SUMMARY

The growth-promoting properties of the proteins of 5 samples of mature cowpea seeds grown in Texas and of 4 samples grown in Oklahoma were measured by incorporating the cowpeas as the source of protein in the diets of young rats. The 9 samples, representing 8 varieties of cowpeas, were used both raw and cooked and 6 of the raw cowpea diets were supplemented with methionine. All diets were fed in a single experiment arranged in a 5×5 balanced lattice design.

The differences in the mean growth responses of the rats to the various raw cowpea diets were not statistically significant. There were, however, indications that the quality of the protein was not the same for all samples of cowpeas and that this variation in quality was associated partially with the variety and partially with the location at which the plants were grown.

Cooking the cowpeas in a double boiler had little effect on the growth-promoting property of some samples but markedly increased the growth response elicited by others. This effect was independent of the location at which the samples were grown.

Supplementing the diets containing the raw cowpeas with 0.3% methionine significantly increased the mean gain responses of the rats over those obtained from the unsupplemented raw or from the cooked samples.

The use of the balanced lattice design resulted in a reduction in the error variance of about 7%. The application of this design to the rat feeding experiment is discussed.

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ZINC METABOLISM OF YOUNG COLLEGE WOMEN ON SELF-SELECTED DIETS

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The function of zinc in the nutrition of man has been studied in an attempt to determine the needs of the body for this element. Drinker and co-workers ('27) were among the early observers of zinc metabolism who used human subjects. They found urinary excretions of zinc not to increase appreciably with high zinc intake, whereas fecal elimination more nearly corresponded with intake.

Two males and one female subject observed by McCance and Widdowson ('42) excreted an average of 0.3 mg of zinc per day in the urine. These authors also state that this amount did not vary with intake by mouth or by intravenous injections, thus suggesting that, in normal life, the kidney can take little or no part in regulating the amount of zinc in the body. Within the limits of experimental error, these subjects excreted almost the same amount of zinc in their stools as they ingested in their food. This is the only study noted where there was not a definite zinc retention.

Three boys between the ages of three and 6 years studied by Scoular ('39) retained between 2 and 3 mg daily on a daily intake of from 4 to 6 mg, the highest ingestion being 41% higher than the lowest in the study. Urinary zinc was from 0.04 to 6% of the intake, this amount not being related to the intake nor constant for a given individual. Forty-two

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to 163% of the ingested zinc was excreted in the feces. Of the 35 balances determined, 10 were negative.

When the average zinc retention for 8 children, aged 8 to 12, was studied by Stern et al. ('41), the amount ranged from 17 to 52.3% of the 15.6 mg of zinc ingested. From 2.6 to 4.2% of the zinc intake was excreted in the urine, with 44.6 to 78.8% being excreted in the feces. This gave an average retention of 4.8 mg daily for these children.

On the basis of these few human zinc metabolism studies it appears that the gastrointestinal tract is the mode of zinc elimination from the body, with the kidney playing a minor role, if any, in retention of the element. No definite pattern of retention has been shown to be established.

No studies have been noted that show the intake, retention and pattern of excretion of zinc for college-age women. Therefore, the present study was undertaken to observe the zinc metabolism of young college women consuming self-selected diets.

EXPERIMENTAL PROCEDURE

Thirteen college women, 17 to 27 years of age, participated voluntarily in this study while living in the Home Management House Duplex. The meals consumed by the two groups of girls were planned cooperatively. Each group, however, purchased and prepared its food separately. Unpublished data show a tendency for small servings of composite foods. Consequently, group 2 was asked to use a concentrated food preparation [2] as a supplement, in preparing its meals. Recipes were provided both groups with suggested ways of supplementing them with the multi-purpose food for group 2.

The technique used in the collection of food and excreta for analysis in the present study was that previously reported by Holt and Scoular ('48). Servings of each food from each meal, similar in all respects to those eaten by the college

[2] Multi-Purpose Food, Formula B. Ingredients: soy grits, salt, fortified with calcium pyrophosphate, kelp, vitamin A ester, irradiated ergosterol, vitamin B_1, riboflavin (B_2) and niacinamide. This was obtained from Meals for Millions Foundation, Incorporated, Los Angeles, California.

women, were collected in weighed glass jars at the same time that the subjects were served at the table. Only aged glassware was used in the collecting and storing of both food and excreta. Milk was analyzed separately in order to permit each girl to consume the amount she desired, by removing daily aliquots to give one homogeneous sample for analysis. A record was kept of the amount of milk consumed by each girl each day, and suitable additions were then made to each individual's record of food intake, to include the milk zinc.

Aliquots of both food and feces were obtained for analysis after being weighed and macerated in the Waring Blendor. Aliquots were removed from the 24-hour urine collections which had been made directly into amber gallon bottles.

One liter of tap water was evaporated to 50 ml, and duplicate tests were run to determine the zinc content of the water supply at the girls' residence. This water contained 0.0072 mg/l, which amount was considered to be negligible in determination of the zinc intake, since all water used in cooking was analyzed with the composite food samples.

Hibbard's ('37) dithizone procedure was used in determining the zinc content, in triplicate, of all food and excreta samples. Every precaution was taken to prevent contamination of the samples prior to and during the analyses. Recoveries from the addition of 2 mg of zinc to food, milk, urine and feces samples ranged from 95.5 to 100.2%.

DISCUSSION OF RESULTS

The average daily intakes and excretions of zinc of 13 young college women are given in table 1. The zinc content of the day's meals was different for each of the 5-day metabolism periods, although only the average for the 5 days is shown in the table. Two of the young women in group 1 had their night meal outside of the house on one of these days. These meals were analyzed separately and suitable corrections were made for their zinc content, giving Co and Ho daily averages of 11.8 mg as compared to 11.5 for the other members of group 1. The use of the food concentrate (M P F)

TABLE 1

Average daily zinc intake and excretion of college women consuming self-selected diets

SUBJECT	AVERAGE DAILY INTAKE			EXCRETION			RETENTION	EXCRETED IN	
	Composite	Milk	Total	Urine	Feces	Total		Urine	Feces
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	%	%
Group 1									
Wi	11.5	0.6	12.1	1.3	3.2	4.5	7.6	11	26
Co	11.8 [1]	0.5	12.3	0.6	4.1	4.7	7.6	6	34
Yo	11.5	0.9	12.4	0.7	5.9	6.6	5.8	6	47
Ho	11.8 [1]	0.9	12.7	1.1	4.1	5.2	7.5	8	32
St	11.5	0.3	11.8	1.8	4.5	6.3	5.5	16	38
Le	11.5	0.6	12.1	1.6	5.2	6.8	5.3	13	43
So	11.5	0.8	12.3	0.6	5.5	6.1	6.2	5	45
Average	11.6	0.7	12.3	1.1	4.6	5.7	6.6	9	38
Group 2									
Mc	12.7	0.8	13.5	0.8	7.6	8.4	5.1	10	56
Fr	12.7	1.4	14.1	0.8	6.3	7.1	7.0	6	45
Ko	12.7	1.4	14.1	1.0	6.7	7.7	6.4	7	47
Bi	12.7	1.4	14.1	0.8	7.9	8.7	5.4	5	55
As	12.7	1.3	14.0	0.9	6.4	7.3	6.7	7	46
Br	12.7	1.2	13.9	0.8	4.3	5.1	8.8	5	31
Average	12.7	1.3	14.0	0.9	6.5	7.4	6.6	7	47
Mean average	12.2	1.0	13.2	1.0	5.6	6.6	6.6	8	42

[1] Night meal eaten outside the house and analyzed separately to obtain this total.

by group 2 not only gave more concentrated composite food samples which weighed more than the same meals prepared by group 1, but also gave slightly higher zinc values.[3] Although the use of the M P F increased the zinc content slightly, the daily variation was not marked. The lowest value, 9.8 mg, was obtained from the food composite of group 1 on the second day, whereas the highest value, 14.4 mg, was obtained with group 2 the first day, giving a difference of 4.6 mg. The use of the M P F by group 2 may have contributed to this difference in zinc content. Multi-purpose food, because of its concentration, gave a larger weight to the composite food sample, without a change in volume. This difference in weight, as it affected the zinc intake, is evidenced by the weights for one day. The weight of the composite food of group 1 on this day was 968.3 gm and it provided 12.6 mg of zinc, while the same menus and volume for group 2 weighed 1,316.8 gm and provided 14.4 mg of zinc. Furthermore, regardless of the use of the M P F, the kind of foods served also made a difference in the daily zinc intake. Beef, eggs, yeast bread and potatoes, which are all good sources of zinc, were served on the days of the highest zinc values.

The subjects of group 2 commented on feeling uncomfortably full after meals and, on the second day, had to eat part of their breakfast in the middle of the morning in order to consume the same volume of food as the subjects of group 1. Apparently this was associated with the concentration of the food consumed, as this volume of food for group 2 weighed 329.4 gm more than the same volume of similar foods consumed by group 1. On the day when the food composite weight increased only 35.3 gm over that of group 1, the subjects of group 2 did not have the uncomfortably full feeling experienced on the other days of the study.

The amount of milk consumed made a noticeable difference in the total zinc intake of these subjects. The fluid milk con-

[3] The M P F composite foods were higher in protein, calcium and phosphorus than the non-M P F composites, namely: 48, 1.2 and 0.7 gm for the former, while the latter furnished 35, 0.86 and 0.40 gm, respectively.

sumed by group 1 ranged from 0.4 to 1.2 cups per day, and from 1 to 1.7 cups for group 2. The average daily milk intake contributed from 0.5 to 0.9 mg of zinc to the total intake of the subjects in group 1 and from 0.8 to 1.4 mg to the intake of those in group 2.

Food and milk composites for the subjects gave an average total daily intake of zinc that ranged from 11.8 to 12.7 mg and from 13.5 to 14.1 mg for the two groups. The average daily zinc intake for group 1 was 12.3 mg and for group 2, 14.0 mg. Thus the difference in the daily averages for the two groups was only 1.8 mg. The zinc intake of these college women was somewhat lower than the 15.6 mg consumed by the 8 children studied by Stern et al. ('41), but higher than the 4 to 6 mg consumed by three pre-school age boys studied by Scoular ('39). However, for college women, the subjects consumed relatively small amounts of food, as previously observed in relation to other studies.

The average daily fecal excretion for group 1 ranged from 3.2 to 5.9 mg, with an average of 4.6 mg for the 7 subjects. The average for group 2 was slightly higher, 6.5, with a range of 4.3 to 7.9 mg for the 6 subjects. The increased fecal excretion of zinc by group 2 was associated with the increased zinc intake of this group. The adult study reported by McCance and Widdowson ('42) found the fecal excretion to almost equal the amounts ingested. This is not true of the adults of the present study.

Individual daily urinary excretion ranged from 0.6 to 1.8 and from 0.8 to 1.0 mg for group 1 and group 2, respectively. These values gave an average daily urinary excretion of 1.1 mg for group 1 and 0.9 mg for group 2. The fact that the slightly higher zinc ingestion of group 2 did not increase the urinary excretion of zinc is in accord with results reported by McCance and Widdowson ('42), who observed urinary excretion not to vary with increased zinc intake.

Five to 16% of the zinc ingested by the subjects of group 1, with an average of 9%, was excreted in the urine. The per cent for group 2 ranged from 5 to 10, with an average of

7%, whereas the fecal excretions for these two groups were from 26 to 47, with an average of 38%, and from 31 to 56, with an average of 47%, respectively. These fecal percentage values are low compared with those reported for children by Scoular ('39) of 42 to 163% and the 44.6 to 78.8% observed by Stern et al. ('41).

Retentions given in this study are based solely upon the difference between the intake and the urinary and fecal excretion. Although the individual retentions varied from 5.3 to 7.6 mg for group 1 and from 5.1 to 8.8 mg for group 2, the average retentions were identical for the two groups, namely, 6.6 mg. This retention is higher than the averages reported by both Scoular ('39) and Stern et al. ('41) for children. On the basis of the present study it appears that college women excrete a larger per cent of ingested zinc in the urine and a smaller per cent in the feces, with retentions higher in proportion to the level of zinc ingested, than do younger subjects.

SUMMARY

Self-selected diets furnished from 9.8 to 14.4 mg of zinc daily.

The subjects excreted from 0.6 to 1.8 mg of zinc in the urine and from 3.2 to 7.9 mg in the feces.

An average of 8% of the ingested zinc was excreted in the urine and 42% in the feces.

All of the subjects were in positive zinc balance and retained an average of 6.6 mg per day.

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THE EFFECT OF INCIPIENT VITAMIN A DEFICIENCY ON REPRODUCTION IN THE RABBIT

I. DECIDUA, OVA AND FERTILIZATION [1]

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SIX FIGURES

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In the course of investigations dealing with the cause of a sterility syndrome in female rabbits associated with soybean hay feeding (Kendall et al., '50), it became necessary for comparative purposes to establish clearly the effect of vitamin A deficiency on the reproductive performance of the female rabbit. Previous work (Evans, '28; Mason, '35) had shown the effect of a severe vitamin A deficiency in laboratory animals. Others have investigated the effect on reproduction of severe vitamin A deficiency in cattle (Hart et al., '24; Guilbert et al., '35, '40; Kuhlman and Gallup, '40, '42; Davis and Madsen, '41; Hilton et al., '44; and many others).

Warkany and his co-workers (Warkany and Schraffenberger, '44, '46; Warkany and Roth, '48) have reported on the appearance of fetal abnormalities and abortions in rats on vitamin A-deficient diets. They have shown that the incidence and severity of the symptoms may be affected by varying the carotene intake. Moore and his associates (Moore

[1] The data reported in this paper were taken from a thesis submitted by the senior author to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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and Sykes, '40; Moore et al., '43, '48) have shown that elevated cerebrospinal fluid pressure, a symptom of vitamin A deficiency in calves, resulted before other physiological impairment and on carotene intakes higher than those necessary for growth. In cattle vitamin A requirements for satisfactory reproduction are higher than for maintenance or for growth (Guilbert et al., '40).

It was our purpose to determine the effect of the development of vitamin A deficiency in female rabbits on their reproductive performance before the appearance of visible symptoms of vitamin A deficiency. This condition of onset of deficiency is referred to here as incipient deficiency.

EXPERIMENTAL

Sixty-four female rabbits of the New Zealand White and of the Dutch breeds were used. All experimental animals except where specifically stated below received ad libitum a carotene-deficient diet modified after that used by Phillips and Bohstedt ('38) for 12 to 14 weeks before mating. It was composed by weight of ground white corn, 22.0 parts; wheat middlings, 11.5 parts; oat mill feed, 40 parts; linseed oil meal, 23.0 parts; steamed bone meal, 3.0 parts; iodized salt, 0.5 parts; a commercial preparation [3] for vitamin D in the amount of 100 gm per 100 pounds of the mixed ration. This mixture contained no detectable carotene, using the method of Moore and Ely ('41). Details of rations, vitamin supplements, and number of animals used in each experiment are given in table 1.

At the termination of each experiment, all animals were anesthetized, blood was removed by heart puncture for analyses, and the animals were killed by rapid intravenous injection of sodium pentobarbital and then immediately autopsied. The state of vitamin A deficiency was determined by liver and blood plasma analyses. The procedure of Gallup and Hoefer ('46) was used for extraction of vitamin A from the

[3] Delsterol.

liver and that of Kimble ('39) for blood. Vitamin A was determined by the procedure of Sobel and Werbin ('45). In the initial experiments, analyses showed that there was no detectable carotene in the blood or livers. The reproductive tracts of all animals were studied.

TABLE 1

The effect of vitamin A deficiency on ova

DIET	NO.	BREED	HOURS PREGNANT	NO. OVULATING	PLASMA VIT. A	LIVER VIT. A	AVERAGE CORPORA LUTEA	AVERAGE OVA RECOVERED	AVERAGE OVA CLEAVED
					μg/100 gm	*μg/gm*			
				Exp. 1					
C.D.[1]	6	D	*[2]	5	2.08	0.76	5.8	..	..
Com.	12	D		10	29.83	38.68	6.7	..	..
				Exp. 2					
C.D.	5	D	40	5	9.25	1.84	6.4	4.8	2.2
Com.	5	D		4	43.00	31.50	6.3	5.8	4.0
				Exp. 3					
C.D.[3]	10	NZW	40	6	5.25	1.00	8.8	7.5	6.8
C.D.[4]	10	NZW		6	47.00	124.50	9.5	9.0	8.7
				Exp. 4					
C.D.[3]	8	NZW	96	4	5.00	1.31	8.3	6.0	4.0
C.D.[4]	8	NZW		7	44.80	63.90	10.3	9.6	9.3

[1] C.D. = carotene-deficient.
Com. = commercial (9.5 mg carotene per pound).

[2] * = 66 hours pseudopregnant.

[3] One hundred milligrams α-tocopherol acetate per week given orally.

[4] Seven thousand five hundred micrograms vitamin A acetate and 100 mg α-tocopherol acetate per week given orally.

Experiment 1, effect on decidua formation

After 12 weeks on the diet the does were mated to a vasectomized male and 30 hours later, under anesthesia, the abdomen was opened and a loose loop of thread inserted by needle through the lumen of the left horn of each uterus. The experiment was terminated 36 hours later. The two horns of each uterus were compared for differences in weight, mean diameter and histological appearance with uteri from castrate

rabbits sensitized with estradiol benzoate and given graded doses of progesterone.

No differences between the groups were observed, decidua formation and uterine proliferation being normal in both. The number of corpora lutea was less in does on the carotene-deficient ration than in does fed adequate amounts of carotene. The difference was significant at the 5% level of probability. Premature nuclear degeneration of ova which was later frequently observed was first noticed in this experiment. A normal unfertilized rabbit ovum 66 hours post coitum is shown in figure 1. Figure 2 shows a tubal ovum with premature nuclear degeneration.

The average plasma and liver levels of the vitamin A-deficient rabbits shown in table 1 do not include those of two of the rabbits which had so little vitamin A that it could not be measured by the standard methods used.

Experiments 2 and 3, effect on ovulation rate and cleavage rate

The animals received the diets and vitamin supplements as indicated in table 1 for 14 weeks before mating. The ova obtained by flushing the oviducts with warm physiological saline 40 hours post coitum were counted and examined for evidence of cleavage. The results are shown in table 1.

Experiment 4, effect on early embryonic development

At autopsy 4 days post coitum, the uterus and oviducts were flushed with warm physiological saline and the contents examined for ova and blastocysts. Figure 3 shows normal and abnormal ova taken from the same vitamin A-deficient rabbit, while figures 5 and 6 show other examples of abnormal ova recovered 4 days post coitum. A normal blastocyst 4 days post coitum is shown in figure 4.

Using the tubal flushing technique, 93.5% of 154 eggs ovulated, as indicated by fresh corpora lutea, were recovered from control animals in all our experiments. However, only

78.7% of 118 eggs were recovered of the corpora lutea counted in the ovaries of females on the carotene-deficient diet. Serial histological sections of the ovaries of 6 animals on the deficient diet indicated that ovulation was complete in each instance, suggesting early degeneration of the ova as a probable cause of failure to recover a higher percentage of the eggs ovulated. While the rabbits fed the carotene-deficient diet in experiment 1 had significantly fewer corpora lutea, small differences in the average number of corpora lutea observed in later experiments were not significant.

Of the eggs recovered from control rabbits 40 hours post coitum, 88.3% appeared to be cleaving normally, compared with 75.4% in the vitamin A-deficient groups. At 4 days post coitum 97% of the eggs in control rabbits appeared as normal blastocysts, compared with only 66.7% in deficient animals.

In all experiments, 7 (24.1%) of the deficient animals produced one or more abnormal eggs in which there appeared to be premature degeneration of the nuclei. The vitamin A levels in these animals varied from 0.54 μg to 2.92 μg (mean 1.21 μg) per gram fresh liver, and from 2.00 μg to 12.00 μg (mean 6.00 μg) per 100 ml blood plasma. The blood plasma of one animal contained so little vitamin A that it was not possible to determine the amount with the methods employed. This value is not included in the mean. No abnormalities were observed in ova from control rabbits. At no time were any symptoms observed with respect to the eyes or haircoat to suggest that the rabbits were severely deficient in vitamin A.

DISCUSSION

Evans ('28) reported that vitamin A deficiency reduced conception rate in rats and resulted in a suppression of pseudopregnancy after sterile mating. He attributed this to a lack of "internal secretions" due to vitamin A deficiency. While Mason ('35) confirmed the reduction in conception rate, he reported that the decidual reaction in rats was not affected by severe vitamin A deficiency. This suggests that

such animals produced enough progesterone to give a normal decidual reaction. The results from experiment 1 indicate that incipient vitamin A deficiency in rabbits did not reduce uterine proliferation in early pregnancy. It is concluded that fertile eggs in such an environment should nidate. However, this experiment did not indicate whether or not sufficient progestins were produced after implantation to maintain young after this time.

Experiments reported here show that ovum abnormalities occur in rabbits fed a carotene-deficient diet at a stage before typical symptoms of vitamin A deficiency are apparent. The reduction in the number of eggs recovered compared to corpora lutea in rabbits with incipient vitamin A deficiency appears in part to be due to early degeneration of ova. A further loss of ova before implantation in such animals occurs due to an increased number of uncleaved eggs at both 40 hours and 4 days post coitum. This reduction of breeding efficiency occurred in animals which appeared healthy. The high incidence of ovum abnormalities and the decreased number of recovered eggs cleaving in rabbits with incipient vitamin A deficiency may account for previous reports where no implantation sites were found in vitamin A-deficient rats after fertile copulation (Parkes and Drummond, '26; Evans, '28).

In a subsequent paper details will be given of the effect of incipient vitamin A deficiency on embryonic and fetal development in similar groups of mature rabbits.

SUMMARY

A study was made to determine the effect of incipient vitamin A deficiency, i.e., the deficiency before visible symptoms occur, on the early stages of reproduction in the female rabbit. The vitamin A deficiency did not reduce decidua formation after the insertion of a thread into the uteri of mated females. The deficiency did, however, produce premature degeneration of ova and reduced the number of fertilized ova at 40 hours and 4 days post coitum.

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PLATE 1

EXPLANATION OF FIGURES

(Approximate magnifications given)

1 Unfertilized ovum from a normal rabbit 66 hours post coitum. × 150.

2 Degenerating unfertilized ovum from a rabbit with incipient vitamin A deficiency, 66 hours post coitum. × 150.

3 Normal blastocysts and abnormal eggs recovered from a rabbit with incipient vitamin A deficiency 4 days post coitum. × 75.

4 Normal rabbit blastocyst 4 days post coitum. × 60.

5 A collapsed or degenerating blastocyst 4 days post coitum from a rabbit with incipient vitamin A deficiency. × 150.

6 Degenerating ovum 4 days post coitum from a rabbit with incipient vitamin A deficiency. × 150.


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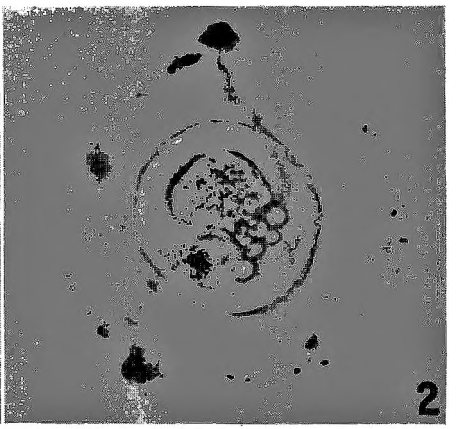
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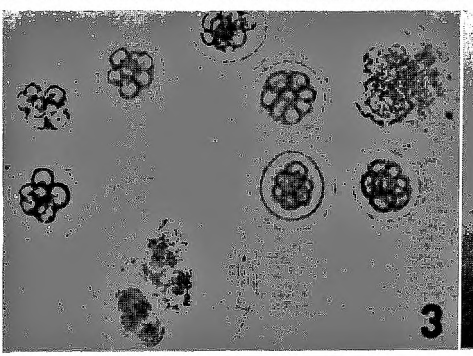
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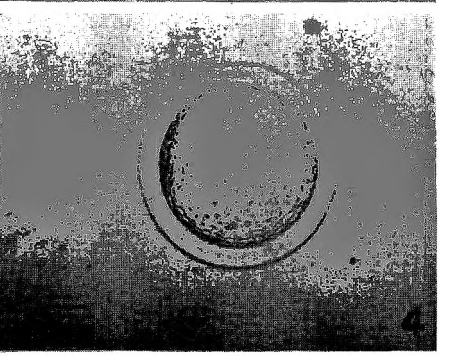
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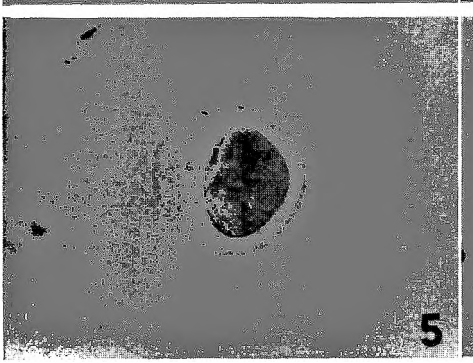
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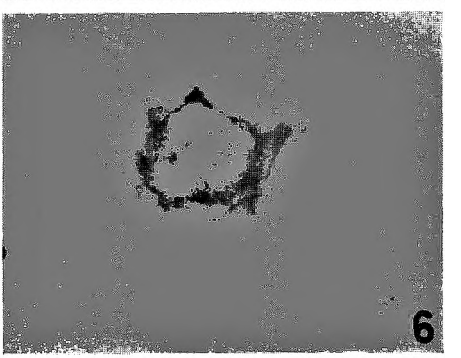
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THE EFFECT OF INCIPIENT VITAMIN A DEFICIENCY ON REPRODUCTION IN THE RABBIT

II. EMBRYONIC AND FETAL DEVELOPMENT [1]

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SEVEN FIGURES

(Received for publication September 18, 1953)

That incipient vitamin A deficiency could adversely affect reproductive performance in the female rabbit before other discernible symptoms were observed was recently reported by Lamming et al. ('54). The previous work, which was confined to studies during the first 4 days of pregnancy, showed that ovum infertility and degeneration in vitamin A-deficient rabbits reduced the average number of viable eggs per pregnant doe. The present report concerns studies to determine whether or not incipient vitamin A deficiency affects embryonic and fetal development as well as litter size.

EXPERIMENTAL

Eighty-four female rabbits of the Dutch and New Zealand White breeds were used. One-half of the rabbits were fed a diet containing no detectable carotene (Lamming et al., '54), while the other half were fed the same diet plus a vitamin

[1] The data reported in this paper were taken from a thesis submitted by the senior author to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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supplement or commercial rabbit pellets (see table 1). The animals were autopsied at varying intervals post coitum and the blood and livers were analyzed for vitamin A. The numbers of corpora lutea, embryos and implantation sites were noted. In the later experiments the embryos or fetuses were weighed.

RESULTS

The results are presented in table 1. In many of the experiments several of the rabbits were not pregnant at autopsy, especially in the vitamin A-deficient group. In the second experiment the fetuses of two deficient rabbits averaged 338 mg. The fetuses of only one control animal were weighed, but they averaged 735 mg. This was the first indication that incipient vitamin A deficiency might reduce embryo weight. Thereafter, all fetuses were weighed individually.

In experiment 3, the 18 fetuses of the three pregnant rabbits on the deficient diet weighed 469 ± 77.1 mg, as compared to 678 ± 54.4 mg for the 45 fetuses of the 4 pregnant rabbits in the control group. This difference is highly significant, even without considering the large difference in litter size.

In a similar trial (exp. 4) all rabbits received 100 mg α-tocopherol acetate weekly. The 20 fetuses of the three pregnant deficient rabbits weighed 505 ± 96.7 mg, as compared to 515 ± 118.9 mg for the 51 fetuses of the 6 pregnant rabbits in the control group.

To determine the effect of vitamin A deficiency on fetal mortality, 14 pairs of mature female litter-mate Dutch rabbits were fed the carotene-deficient diet and one animal of each pair received, in addition, the vitamin A supplement (exp. 5). After 14 weeks the rabbits were mated at convenient intervals to the same Dutch male. All rabbits receiving the vitamin A supplement were pregnant at autopsy. Of the 12 vitamin A-deficient animals which mated, two failed to show evidence of implantation 28 days post coitum, 5 aborted or partially resorbed their uterine contents from the 16th to 23rd day of gestation (fig. 2), and one had 5 resorbing fetuses at au-

TABLE 1

The effect of incipient vitamin A deficiency on abortion and resorption in the rabbit

EXPERIMENT NUMBER	DIET	NO.	BREED	DAYS PREGNANT	NO. PREGNANT	PLASMA VIT. A	LIVER VIT. A	AVERAGE CORPORA LUTEA	AVERAGE LIVING FETUSES	AVERAGE RESORPTION SITES
						μg/100 ml	*μg/gm*			
1	C.D.[1,3]	8	NZW	10	2	14.00	1.36	8.5	7.0	. .
	C.D.[4]	8	NZW	10	5	79.20	108.70	8.4	7.8	. .
2	C.D.	5	D	16	3	a[5]	a	7.6	6.0	0.7
	Comm.	5	D	16	3	40.00	34.73	7.3	6.7	0.0
3	C.D.	5	NZW	16	3	a	a	9.3	6.0*[6]	0.0
	C.D.[2]	5	NZW	16	4	64.25	16.67	13.5	11.25*	0.0
4	C.D.[3]	10	NZW	16	3	8.00	1.40	8.7	6.7	1.0
	C.D.[4]	10	NZW	16	6	55.50	152.84	9.7	8.5	0.7
5	C.D.	14	D	16–28	10	4.00	0.55	7.2	1.9**	3.3**
	C.D.[2]	14	D	16–28	14	16.79	29.07	8.1	6.1**	0.5**

[1] C.D. = carotene-deficient. Comm. = commercial (9.5 mg carotene per pound).

[2] Seven thousand five hundred micrograms of vitamin A acetate per week given orally.

[3] One hundred milligrams α-tocopherol acetate per week given orally.

[4] One hundred milligrams α-tocopherol acetate and 7,500 μg vitamin A acetate per week given orally.

[5] a = Less than 4.0 μg vitamin A per 100 ml blood plasma and less than approximately 0.5 μg vitamin A per gram liver.

[6] * = Difference significant at the 5% level of probability.

** = Difference significant at the 1% level of probability.

topsy, 28 days post coitum (fig. 1). These animals were sacrificed as the symptoms of abortion or resorption appeared and the sister control rabbits were killed at the same time for comparison. The remainder of both groups were killed 28 days post coitum. Each fetus was examined and weighed. The 85 fetuses of the 14 control rabbits averaged 30.94 $\pm$ 5.21 gm live weight, while the 19 fetuses of the 4 pregnant rabbits of the vitamin A-deficient group averaged 26.17 $\pm$ 4.84 gm. The differences in number of normal fetuses per pregnant doe and in the weight of normal fetuses were significant at the 1% level of probability.

Ocular abnormalities were observed in fetuses from two deficient animals autopsied 28 days post coitum (figs. 3 and 4). No histological examinations were made with respect to this defect. However, the abnormalities noted appeared similar to the "open-eye" condition reported by Warkany and co-workers ('44, '46, '48) in fetuses from rats with severe vitamin A deficiency. Many other fetuses appeared to have malformation of the vitreous body, over which the eyelids fused normally.

In three fetuses from two deficient rabbits the abdominal wall failed to enclose completely the viscera (fig. 6). It is not known whether or not this condition was a result of the deficiency.

Many fetal placentae from deficient rabbits which aborted or were autopsied 28 days post coitum were light colored, with a gross appearance of a reduced vascularity. Figure 5 shows a normal fetal placenta and an abnormal placenta from a vitamin A-deficient rabbit. The corpora lutea in the ovaries of deficient rabbits appeared normal by macroscopic examination (fig. 7).

Analysis of the data from all experiments reported in this and the previous report (Lamming et al., '54) is further evidence that incipient vitamin A deficiency adversely affects reproduction in rabbits. These data, presented in table 2, show that the vitamin A deficiency not only reduced the number of females which accepted the male but also reduced the

per cent of the mated rabbits which were pregnant. This reduced the number of conceptions in vitamin A-deficient rabbits, as indicated by active corporea lutea, by 18% when compared to similar animals receiving adequate vitamin A. The average number of corpora lutea per pregnant animal was not significantly affected by incipient vitamin A deficiency in either breed used.

TABLE 2

The effect of incipient vitamin A deficiency on estrus and the number of pregnancies following mating

CATEGORY OF INTEREST	VITAMIN A-DEFICIENT RABBITS	CONTROL RABBITS
Total number available for mating	88	83
Total number mated	68	76
Percentage of total mated	77.3	91.6
Total number pregnant or pseudopregnant	56	68
Percentage of total rabbits pregnant or pseudopregnant	63.6	81.9
Percentage of mated rabbits pregnant or pseudopregnant	82.4	89.5
Average number of corpora lutea per pregnant rabbit		
Dutch breed	6.92 ± 1.32 [1]	7.29 ± 1.67 [1]
New Zealand White breed	9.00 ± 1.93 [1]	10.03 ± 2.70 [1]

[1] Not significantly different.

Where similar groups of rabbits were used, comparisons between experiments at various stages of gestation further illustrate the severity of the effects of incipient vitamin A deficiency at each stage of gestation studied. These data are presented in table 3.

Throughout the experiments, no gross abnormalities were observed at autopsy in the condition of the intestine, heart, liver, kidneys or stomach mucosa in either deficient or control rabbits. At no time were symptoms of the eyes or haircoat observed to suggest that the animals were severely deficient

TABLE 3

Combined results at autopsy of incipiently vitamin A-deficient and control rabbits at various stages of gestation

(Number of animals involved given in parentheses)

CATEGORY OF INTEREST	VITAMIN A-DEFICIENT RABBITS	CONTROL RABBITS
Percentage of eggs recovered compared to fresh corpora lutea 40 hours post coitum [1]	82.2 (13)	94.8 (14)
Percentage of recovered eggs cleaving 40 hours post coitum [1]	78.4 (13)	92.2 (14)
Percentage of corpora lutea represented by normal eggs 40 hours post coitum [1]	64.5 (13)	87.4 (14)
Percentage of eggs recovered compared to fresh corpora lutea 4 days post coitum	72.0 (4)	93.0 (7)
Percentage of recovered eggs cleaving normally 4 days post coitum	66.7 (4)	97.0 (7)
Percentage of corpora lutea represented as implantation sites 10 days post coitum	84.4 (2) [2]	92.8 (5)
Percentage of corpora lutea represented as normal fetuses 16 days post coitum [1]	72.7 (9)	83.6 (13)
Percentage of corpora lutea represented by normal fetuses 28 days post coitum	26.4 (10)	74.6 (14)
Average litter size 16 days post coitum		
Dutch rabbits	6.00 ± 1.00 (3)	6.67 ± 0.18 (3)
New Zealand White rabbits	6.33 ± 1.64 (6) [3]	9.60 ± 2.50 (10) [3]
Average number of normal fetuses per pregnancy 28 days post coitum		
Dutch rabbits	1.90 ± 1.53 (10) [3]	6.07 ± 2.03 (14) [3]

[1] The data for the two breeds are combined in these comparisons because it was found that the two breeds did not differ significantly in their response to incipient vitamin A deficiency.

[2] See experiment 1.

[3] Significant differences at the 1% level of probability.

in vitamin A. Thus the reproductive abnormalities appeared before external symptoms of the disease or gross pathological changes occurred.

DISCUSSION

The results reported in this and the previous paper illustrate the importance of an adequate store of vitamin A for the optimum breeding efficiency of mammals. They show that the external appearance of an animal is not a satisfactory criterion of a sufficient body store of vitamin A for reproduction.

Supplementation with α-tocopherol of vitamin A-deficient rabbits did not affect the appearance of ovum abnormalities due to incipient vitamin A deficiency, nor prevent the high incidence of embryonic mortality. However, it resulted in higher average blood plasma and liver vitamin A values in rabbits fed the carotene-deficient diet for a standard period, probably due to the vitamin A-sparing action of vitamin E as reported by Hickman et al. ('42) and Lemley et al. ('47). This may account for the insignificant differences in average fetus weight between treatments at 16 days post coitum where the animals received α-tocopherol, compared to a highly significant difference in fetus weight where neither vitamin A-deficient nor control animals received additional α-tocopherol.

Comparisons of the effect of incipient vitamin A deficiency at various stages of gestation show that although reproductive efficiency is affected at all stages of gestation, the effects are more severe either before implantation due to ovum infertility or late in gestation due to abortion and fetal atrophy. The fact that the per cent of corpora lutea represented by living fetuses at 10 and 16 days post coitum was higher than would be expected on the basis of the data from animals killed 40 hours post coitum, is probably a reflection of the number and vitamin A status of the animals involved.

While Hammond (cited by Barcroft, '47) reported little effect, in rabbits, of litter size on average fetus weight before the 16th day of pregnancy, the present observations show that average fetus weight at both 16 and 28 days post coitum

may be significantly affected by the nutritional status of the dam. It appears that incipient vitamin A deficiency may retard normal growth of the embryo and fetus, even though the dam appears in good health.

In most cases the abortion and resorption in deficient animals resulted in a rapid decrease in body weight. However, the 4 deficient animals which were pregnant at 28 days post coitum were only 8% lighter than their sister controls. Thus it is improbable that the significant reduction in fetus weight in these animals compared to fetuses from their sister controls can be attributed to inanition in the deficient rabbits. Further experiments in which the feed intake is carefully checked will help to clarify the effect of any differences in feed intake.

In many cases where the deficient animals maintained the pregnancy to the 28th day post coitum the fetuses appeared normal but the fetal placentae had the typical mottled appearance. These observations support the work of Mason ('35), who stated that vitamin A deficiency affected the decidua before affecting the fetus. However, in this study no histological examinations were made of placentae from deficient rabbits. Whether or not this defect involved an impairment of the placental transfer of available nutrients was not revealed by these experiments.

It is probable that vitamin A is essential for both intra-uterine and extra-uterine growth. This function may account for the significant decrease in fetal weight during vitamin A deficiency. However, it cannot adequately account for the syndrome of abortion and resorption during late gestation. This and other work (Mason, '35) has shown that the placenta is the first tisue to exhibit visible impairment during late gestation in animals deprived of adequate vitamin A.

Many of the symptoms of abortion and resorption in incipiently vitamin A-deficient rabbits closely resemble those due to lack of progesterone (Allen and Corner, '29; Pincus and Werthessen, '38). The importance of a continued adequate supply of endogenous progesterone cannot be overlooked. It is possible that vitamin A deficiency may result

in degeneration of the placenta which, in turn, limits the supply of endogenous progesterone, thus causing abortion and resorption late in gestation. The results of experiments testing the relationship of progesterone to the characteristic symptoms described here will be reported later.

Considering the syndrome of vitamin A deficiency during gestation as a whole, the condition of abortion and resorption during late gestation probably represents a more advanced condition of hypovitaminosis A. The less severe symptoms of ovum degeneration, ovum infertility and impaired fetal nutrition are not visible in the intact animal. However, in multiovulatory species they could result in loss of young, while in uniovulatory animals pregnancy would not occur.

SUMMARY

Studies of various stages of gestation in mature female rabbits were made to determine the effect of low levels of vitamin A on the reproductive efficiency of rabbits even though they appear healthy.

1. Incipient vitamin A deficiency reduced by 14% the number of rabbits which mated normally. There was a decrease of 18% in the total number of deficient animals which conceived, compared to the conception rate in similar groups of rabbits receiving adequate vitamin A. However, incipient vitamin A deficiency did not reduce the average number of corpora lutea in pregnant animals at autopsy.

2. Ovum infertility and degeneration in rabbits with incipient vitamin A deficiency resulted in a loss of ova before implantation. Thus the litter size was significantly reduced, as was indicated when deficient animals were autopsied 16 days post coitum.

3. Incipient vitamin A deficiency resulted in a syndrome of resorption and abortion during late gestation. This reduced the number of normal fetuses present per rabbit at 28 days post coitum. It was estimated that only 26.4% of the original ovulations resulted in normal fetuses in deficient rabbits 28 days post coitum, compared to 74.6% in controls. Ocular

abnormalities occurred in fetuses from deficient rabbits, as well as changes in the fetal placentae which involved a mottled appearance suggestive of a decreased vascularity.

4. Impaired embryonic and fetal nutrition in deficient rabbits significantly reduced fetus size at 16 and 28 days post coitum, even though the dams appeared in good health.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. N. L. VanDemark for help in this work, and to Mr. Fay Campbell and his staff at the Dairy Farm for care of the experimental animals. Also, they wish to thank Funk Brothers Seed Company, Bloomington, Illinois, for the generous supply of white corn used in diet formulation in the study.

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———— 1946 Congenital malformations induced in rats by maternal vitamin A deficiency. Arch. Ophthalmol., *35:* 150–169.

PLATE

PLATE 1

EXPLANATION OF FIGURES

(Approximate magnifications given)

1 Uterus of a deficient rabbit 28 days post coitum, showing resorption sites of 5 fetuses. × 0.5.

2 Uterus of a deficient rabbit 20 days post coitum, showing the old placental sites, and hemorrhagic contents of the uterus. × 0.5.

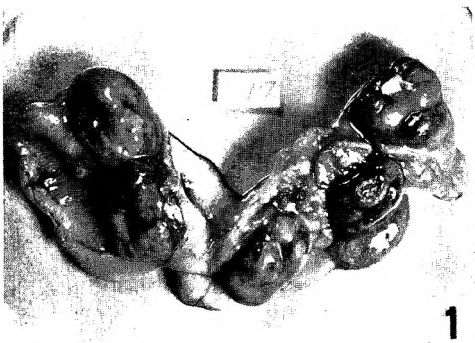
3 Fetus from deficient rabbit 28 days post coitum, showing the mottled fetal placenta and the "open eye" of the fetus. × 1.0.

4 Fetus from a deficient rabbit 28 days post coitum, showing the ocular abnormality. × 1.8.

5 Comparison of a normal vascular fetal placenta and an abnormal mottled placenta. Deficient rabbit autopsied 28 days post coitum. × 1.0.

6 The mottled surface of a fetal placenta 28 days post coitum. Note that the body wall of this fetus did not enclose the intestines. × 1.8.

7 The ovaries of a deficient rabbit 28 days post coitum, showing the prominent corpora lutea and well-developed follicles. × 1.25.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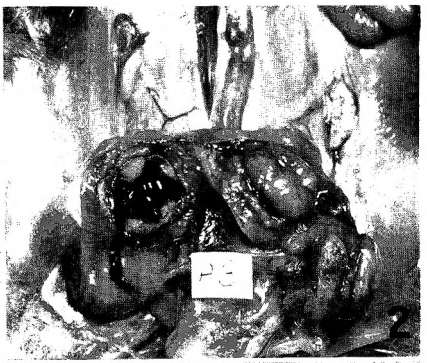
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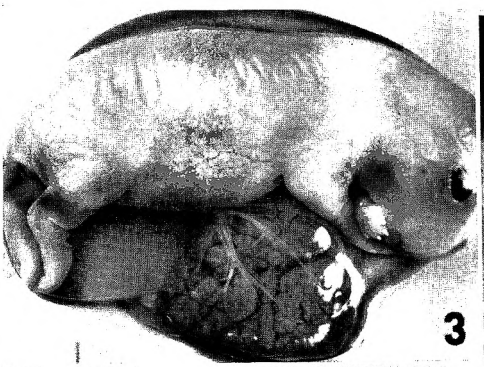
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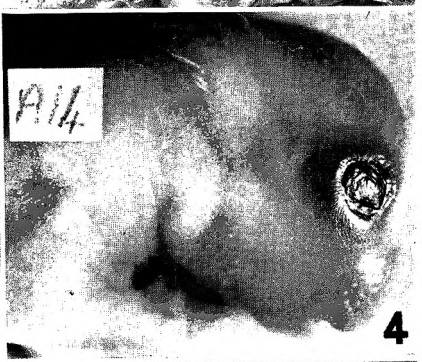
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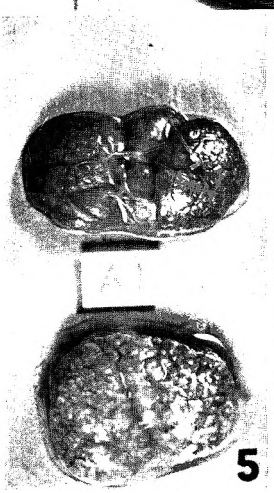
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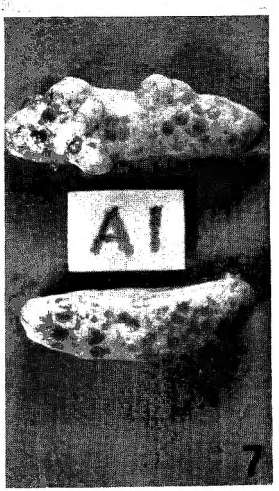
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GROWTH AND FOOD CONSUMPTION IN RELATION TO DIETARY LEVELS OF PROTEIN AND FIBROUS BULK

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FIVE FIGURES

(Received for publication September 8, 1953)

The decrease in the growth rate of chicks that accompanies the feeding of diets containing such fibrous feedstuffs as alfalfa meal or mill feeds has generally been attributed to a reduction in available energy. Yet it is well known that feeding moderate levels of ground corn cobs, oat hulls or washed cellulose may actually increase the growth rate above that obtained with basal diets. Davis and Briggs ('47) replaced glucose in a purified diet with rice hull cellulose [1] at levels of 5, 10 or 15% and found better growth on these diets than on the basal diet that contained no cellulose. These authors suggested that this effect as well as a similar result with sawdust (Davis and Briggs, '48a) might be explained as a specific effect of cellulose or of some cellulose decomposition product. Hoelzel and Carlson ('47) have questioned this interpretation. Davis and Briggs ('48b, c) later observed a similar growth response to levulinic acid or to furfural. Lepp, Harper and Elvehjem ('49) found that with a similar purified diet there was no growth stimulation with levulinic acid, but that either casein, DL-methionine or wood pulp cellulose (cellu flour) increased the growth rate.

[1] Ruffex.

Hill and Dansky ('50) reported that normal growth could be obtained when the energy content of the ration was reduced by adding oat fiber. This was true even when the protein level was below the 20% usually accepted as optimum. They stated that "the productive energy value of the ration was a major factor in controlling food intake." The protein requirement for maximum growth appeared to be a relatively constant, absolute quantity, related to productive energy value through its influence on feed intake. Hill and Dansky ('51) fed oat hulls in levels of from 0 to 40% and found that with each increment of oat hulls up to a level of 20%, an increase in food consumption occurred. Even with levels of oat hulls as high as 40%, growth was not seriously depressed. Differences in body fat content were noted, however, with progressively lower fat contents at higher levels of oat hulls.

Thus it would appear that chicks are able to cope with diets which are quite high in bulk or fiber without a depressive effect on growth.

Diets that contain more than about 5% of certain alfalfa meals, or somewhat higher levels of wheat milling by-products, will not allow normal growth. For alfalfa, at least, a nonfibrous component is responsible (Peterson, '50). The data with mill feeds are less clearcut, but Senior and Kearney ('47) reported that rations containing 50 to 85% of pollard (fine wheat bran) in the diet caused extensive mortality in chicks, whereas the same levels of oats did not, even though both pollard and oats contained about 10% crude fiber. When the fiber obtained by extracting the pollard was fed in combination with wheat flour, low mortality and reasonable gains were obtained.

The experiments reported here are concerned with the effects on growth, food consumption and nutrient utilization obtained by feeding varying levels of protein and of bulk in the form of cellu flour.

METHODS

Single-Comb White Leghorn chicks were maintained on the stock laboratory diet in thermostatically controlled, electrically heated battery brooders until 9 days of age, at which time they were uniformly distributed by weight into groups of 10 chicks. The chick weights and food consumption were determined every other day for 20 days. At the conclusion

TABLE 1

Supplements to diets

INGREDIENT	% OF DIET	INGREDIENT	% OF DIET
Choline chloride	0.2	Calcium carbonate	1.75
2-Methyl-1,4-naphthohydroquinone diacetate	0.001	Tricalcium phosphate	1.8
		Monosodium phosphate	1.3
Thiamine HCl	0.0005	Potassium chloride	0.6
Riboflavin	0.0005	Magnesium sulfate	0.24
Pyridoxine HCl	0.0005	Sodium chloride	0.35
Nicotinic acid	0.0015	Sucrose [4]	1.0
Calcium (d) pantothenate	0.0015	Manganese sulfate	0.015
Biotin	0.00001	Sodium silicate	0.11
Folic acid [1]	0.0002	Ferric citrate	0.003
Vitamin B_{12} [2]	(5 μg/kg)	Cupric sulfate	0.0013
Fortified fish oil (2,250 A — 300 D/ gm)	0.25	Zinc sulfate	0.0013
		Cobalt acetate	0.0006
Natural mixed tocopherols [3]	0.05	Aluminum sulfate	0.003
Crude soybean oil	2.0		

[1] Folic acid was provided by the Lederle Laboratories.
[2] Vitamin B_{12} was provided by Merck and Company.
[3] Natural mixed tocopherols (15%), Distillation Products Industries.
[4] Sucrose was used as a carrier for the vitamin mixture.

of experiment 1, some of the chicks (5 chosen at random from each of the groups fed diets containing cellu flour at the 0, 12 and 24% levels) were analyzed for moisture, crude fat and crude protein (N $\times$ 6.25). These analyses were based on the empty weights of the birds (body weight minus the gastrointestinal tract contents).

The constant ingredients of the experimental diets are shown in table 1.

The variable ingredients in experiments 1 and 2 were glucose,[2] cellu flour [3] and a deboned, acetone-extracted fish meal of excellent protein quality as determined by chick assay methods. In experiment 1 this fish meal was used to provide protein levels of 5.3, 10.7, 16.0 and 21.3%. At each protein level cellu flour was fed at levels of 0, 12, 24, 36 and 48%, and the diets made up to 100% with glucose.

In experiment 2 the protein levels were 15 and 20%, with cellu flour at 0 and 12% for each protein level. Otherwise the diets were the same as in experiment 1, with the exceptions of the supplements discussed below under experiment 2.

In experiments preliminary to those discussed below it was found that on a low-protein diet the rate of growth could be increased by the addition to the diet of either protein or cellu flour. It was apparent that a considerable amount of bulk in the form of cellu flour was well tolerated under conditions of ad libitum feeding. When the energy level per unit weight of diet was reduced, the chicks increased their food intakes.

Experiment 1

This experiment was designed to allow a careful consideration of the relationships among protein level, bulk and the metabolizable carbohydrate level of the diet. The fishmeal used was a complete protein as determined by a separate chick assay. The remainder of the diet was designed to provide a full complement of the known essential nutrients. High levels of vitamins were used in order to provide adequate amounts regardless of the level of food consumption. Under these conditions food consumption was related solely to dietary levels of bulk, protein and energy.[4]

[2] Cerelose.

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

[4] In the discussion of the energy content of the diets, reference is made to the concept of metabolizable energy. This has been estimated as 4 cal. per gram for protein, 3.8 for glucose and 9.0 for fat. Cellu flour is considered merely as bulk and not as a source of energy, an assumption that appears to be borne out by the data.

In figure 1, the growth rates [5] have been plotted against dietary levels of cellu flour at the 4 protein levels. As increasing amounts of cellu flour replaced glucose at the 5.3% protein level, the growth rate increased until maximum growth for this protein level was attained at 36% cellu flour. Even the best growth was much lower than the maximum growth obtained with complete diets. At 10.7% protein, maximum growth was obtained at a level of 24% cellu flour. Normal growth was not produced here, either. With 16% protein diets, maximum growth (6.6% per day) was obtained at a level of 12% cellu flour. Addition of 12% cellu flour produced only a slight growth stimulation at the 21.3% protein level. At each protein level, very high levels of cellu flour resulted in decreased growth rates.

Food consumption

The manner in which variation in the dietary levels of cellu flour and protein affect the food consumption is shown in figure 2. In order to explain the results obtained, it is necessary to consider the sequence of events from the beginning of the experiment. It is assumed here that food is consumed primarily to fulfill the need for energy, as suggested by Hill and Dansky ('50). The average weights of all groups of birds were virtually identical, hence the energy needs of all groups were the same. During the first eating period after being placed on the diets, birds fed diets lacking cellu flour ate sufficient food to satisfy their immediate energy needs. The group eating the diet containing 10.6% protein ingested more protein per unit of energy consumed than did the group receiving 5.3% protein in the diet. This greater intake of

[5] The growth rate was calculated as the average gain per day during the experiment divided by the average of the initial and final body weights. This calculation is useful because it allows comparisons between experiments independent of small variations in body weight.

The growth and food consumption data used here were taken on the 10th day. The birds were continued on the diets for 10 additional days, but although the growth curves followed the same pattern throughout the experiment, mortality in some groups made accurate calculation of nutrient intake impossible after 10 days.

protein allowed greater synthesis of body protein (i.e., more growth), with a resulting increase in energy needs (table 2). Thus as the level of protein was raised, protein synthesis (growth rate) increased until a maximum was reached at the 21.3% level of protein (fig. 1).

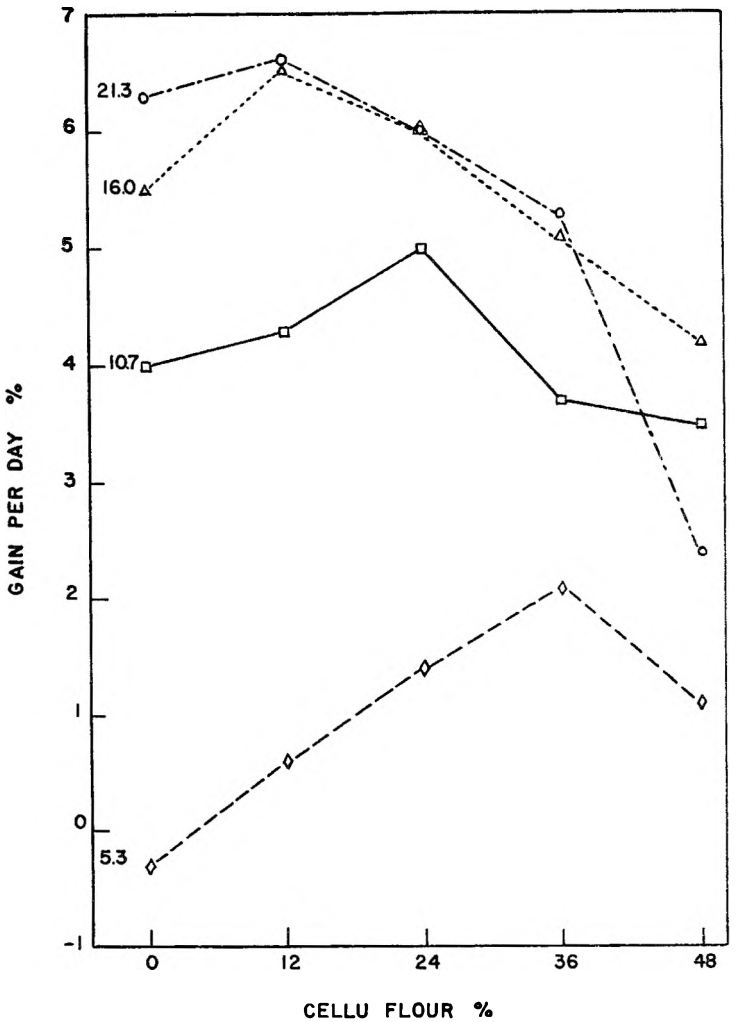

Fig. 1 Growth in relation to dietary levels of protein and cellu flour. (Figures adjacent to curves are protein levels.)

When the birds were fed diets containing 12% cellu flour, their need for energy stimulated food intake as compared with groups given no cellu flour (fig. 2). This resulted in an increased protein intake, which in turn caused a greater energy need. Thus growth was greater with 12% cellu flour than with 0% cellu flour, although the difference became small at 21.3% protein (fig. 1). These same factors operated when the diet contained 24% cellu flour and 5.3% or 10.7%

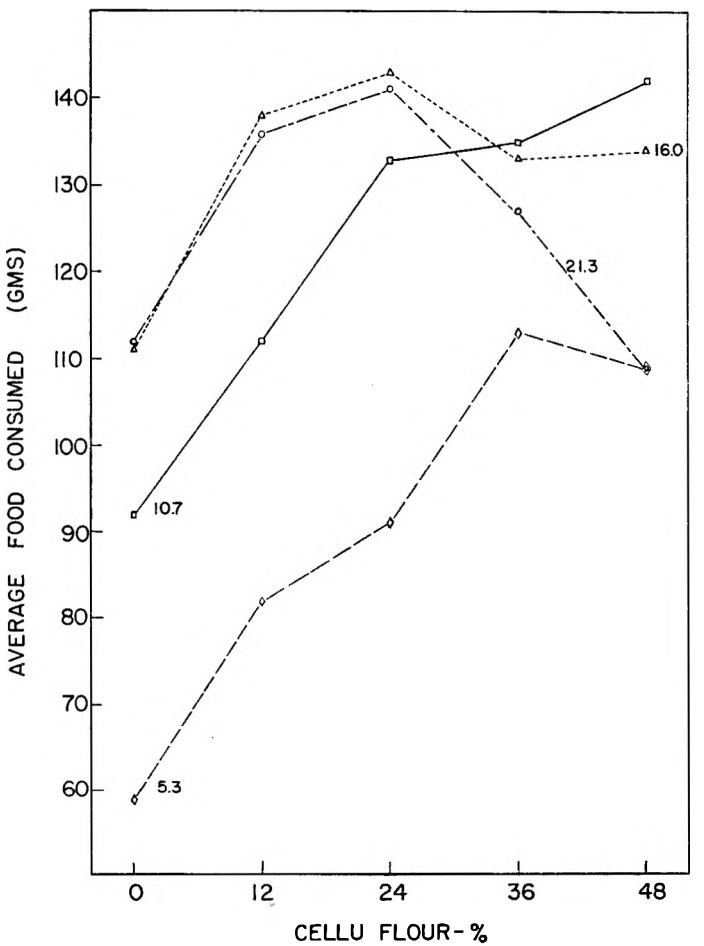

Fig. 2 Food consumption by weight in relation to dietary levels of protein and cellu flour.

protein and when the diet contained 36% cellu floor and 5.3% protein. Thns in the above-cited situations, *the protein intake limited the need for food energy by limiting growth.*

As cellu flour and protein levels were increased, a second major limitation on food consumption became apparent: *the*

TABLE 2

Effects of varying the protein and Cellu flour levels of the diet

(Experiment 1)

PROTEIN	CELLU FLOUR	AVE. PROTEIN CONS'D.	AVE. CALORIES CONS'D.	AVE. BODY WT.	WT. GAIN/ PROTEIN CONS'D.	GAIN/FOOD
%	%	*gm*		*gm*		
5.3	0	3.1	195	57	— 0.50	— 0.03
5.3	12	4.4	237	62	+ 0.80	+ 0.04
5.3	24	4.8	226	66	1.75	0.09
5.3	36	6.0	233	70	2.23	0.12
5.3	48	5.8	180	65	1.17	0.06
10.6	0	9.8	303	85	2.88	0.31
10.6	12	12.0	323	89	2.63	0.28
10.6	24	14.2	328	96	2.70	0.29
10.6	36	14.4	277	85	1.91	0.23
10.6	48	15.2	233	83	1.63	0.22
16.0	0	17.8	363	100	2.43	0.39
16.0	12	22.1	395	115	2.60	0.42
16.0	24	22.9	349	109	2.20	0.35
16.0	36	21.3	270	95	1.81	0.29
16.0	48	21.5	216	89	1.43	0.23
21.3	0	23.8	364	111	2.23	0.47
21.3	12	29.0	385	114	1.93	0.41
21.3	24	30.1	341	109	1.64	0.35
21.3	36	27.1	254	96	1.51	0.32
21.3	48	23.3	176	71	0.69	0.15

weight of food that was eaten reached a maximum (fig. 2). At 16% protein, for example, the weight of food consumed was only slightly greater when the diet contained 24% cellu flour than when it contained 12% cellu flour; however, the volume of food consumed was considerably greater (fig. 3). Since the food energy density was lower at the higher level

of cellu flour, the consumption of energy decreased (table 2), and the growth rate also decreased (fig. 1). This reasoning may also be applied to the 36% cellu flour level at protein levels of 10.7, 16.0 and 21.3% and to the 48% cellu flour level at protein levels of 10.7 and 16.0%.

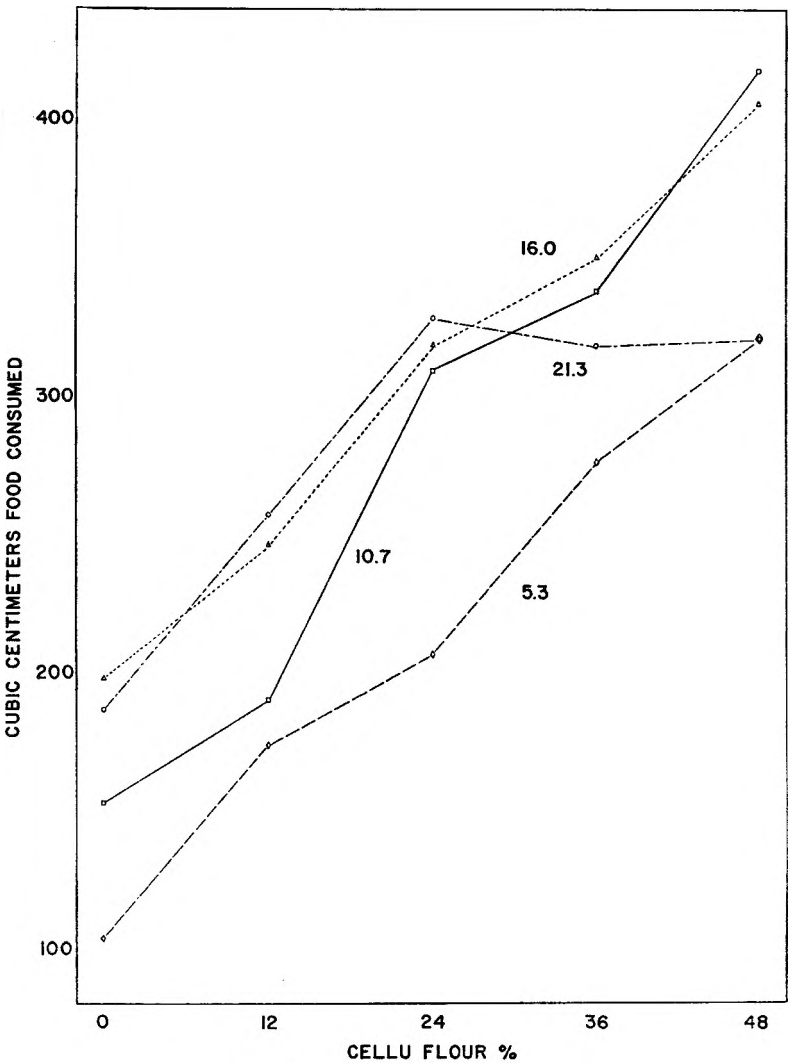

Fig. 3 Food consumption by volume.

At the highest level of cellu flour studied (48%), two factors appeared to limit food intake: at 5.3% protein, body size was small because of a protein deficiency, and the *volume* that was ingested was probably at a maximum (fig. 3); at 21.3% protein, the bulk of the diet was so great in relation to its energy content that an energy deficiency resulted; hence body size was also small. It is interesting to note that the chicks had a capacity to adapt to quite high levels of bulk without diminishing their growth rates.

Growth

The differences in growth rates observed on the diets containing various levels of protein and cellu flour resulted primarily from differences in food consumption. The fact that substitution of 12% cellu floor for glucose increased the growth rate is not startling when the data on food consumption are studied, particularly in relation to amounts of protein and energy consumed (table 2). The growth rate was not maximum unless certain minimum amounts of protein and adequate energy were ingested. Thus growth is limited via food intake by two major factors: the consumption of protein and the consumption of energy. These, in turn, depend upon the protein level of the diet and the energy level of the diet.

Criteria of food utilization

Diets that contained only 12% cellu flour produced about the same ratio of *weight gain* to *food consumed* as diets that contained no cellu flour (table 2), but at the three highest protein levels the ratio gain/food decreased as the bulk was further increased. At the lowest protein level (5.3%), a maximum gain/food ratio was reached at a 36% level of cellu flour.

Since cellu flour must be considered only as bulk and not as a source of nutrients (Hoelzel and Carlson, '47), estimates were made of the ratio of *gain in weight to energy consumed*, and these were plotted (fig. 4) against the level of cellu flour. This ratio increased at the lowest protein level (5.3%) to a maximum at 36% cellu flour and then decreased slightly. The ratio tended to be constant at 10.7% protein and at the

two highest levels of protein (16.0 and 21.3%) became constant and coincided, thus demonstrating that the addition of cellu flour had no effect on food utilization, except in the extreme cases. These exceptions are at a very low level of protein (5.3%) and at a high level of protein when the diet

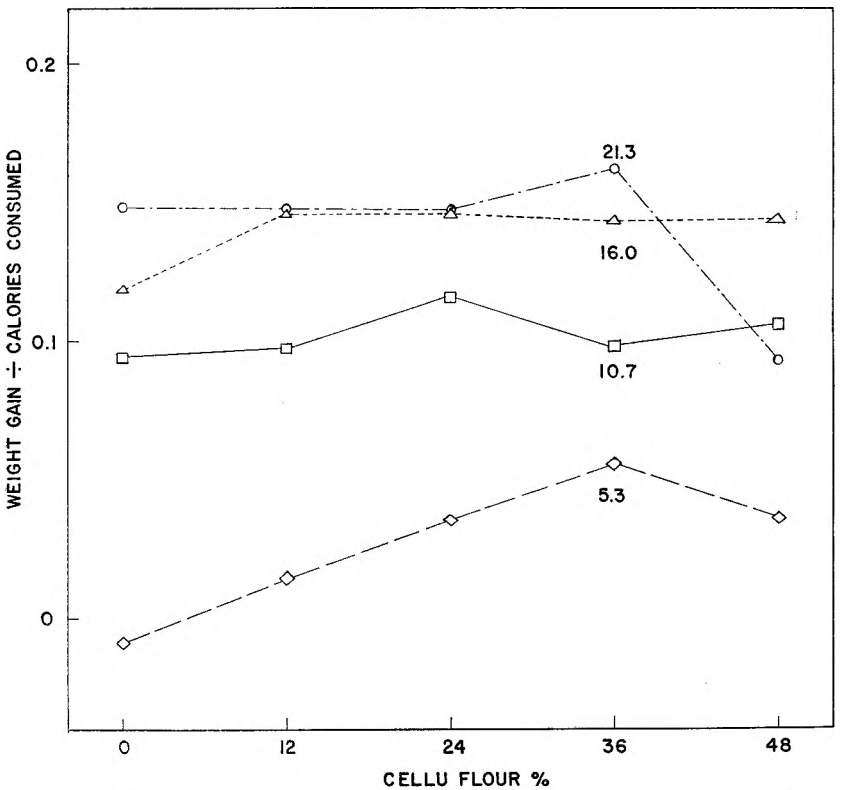

Fig. 4 Relationships of growth, energy consumption and diet composition.

contained 48% cellu flour. The latter instance, as discussed previously, is one in which body weight was comparable with that of the groups on the low-protein diets and consumption of energy was the lowest of all groups (table 2). These data indicate that, with diets containing appreciable amounts of bulk, gain/food is not a satisfactory criterion of food utilization.

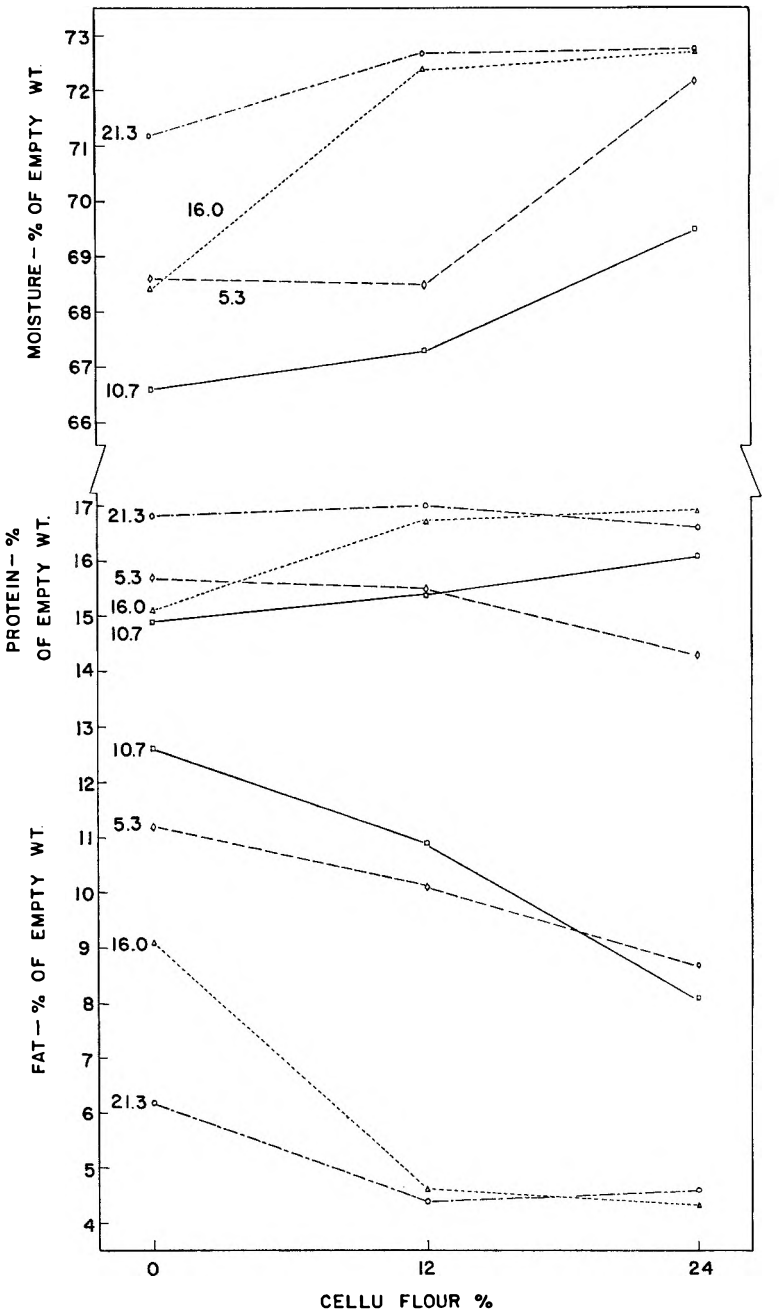

Fig. 5 Body composition as influenced by dietary levels of protein and cellu flour.

The protein content of the carcasses varied only from 2 to 3% among all the groups analyzed (fig. 5), hence weight gain was a good measure of body protein increment. Thus the ratio of gain in weight to protein consumed was an adequate criterion of protein utilization. This ratio is charted

TABLE 3

Weight gains and food consumption with various supplements at two protein levels

(Experiment 2)

PROTEIN LEVEL	SUPPLEMENTS	AVE. GAIN IN WT., 11 DAYS	AVE. GAIN PER DAY	AVE. FOOD CONSUMED
		gm	*%*	*gm*
15	None	54	5.2	114
15	12% Cellu flour	68	6.1	170
15	2% Ca gluconate	59	5.5	147
15	12% Cellu flour 2% Ca gluconate	74	6.5	167
15	0.1% Levulinic acid	55	5.3	133
15	12% Cellu flour 0.1 Levulinic acid	71	6.3	161
20	None	72	6.4	147
20	12% Cellu flour	73	6.4	160
20	2% Ca gluconate	66	6.0	141
20	12% Cellu flour 2% Ca gluconate	70	6.2	164
20	0.1% Levulinic acid	70	6.2	148
20	12% Cellu flour 0.1% Levulinic acid	69	6.1	163

against the cellu flour level of the diet in table 2. Protein efficiency was lowest at the 5.3 and 21.3% protein levels and greatest at intermediate levels. At any protein level, a point is reached (as the glucose level is decreased) where this ratio begins to decline. When the data shown in figure 5 were used to calculate the actual ratio of increase in body

protein as related to protein intake (efficiency of protein utilization), the results obtained showed the same relationships as were shown by the first three groups at each protein level in table 2. However, since only 5 birds from each group were analyzed and all the diets were not represented, these data are not presented here.

The fat content of the carcasses was high on the two lower protein levels and was lower with higher levels of cellu flour. On the two higher protein levels, the fat content was highest at high dietary levels of carbohydrate and lowest at high levels of cellu flour. Moisture content showed an inverse relationship to fat content with all diets (fig. 5).

Experiment 2

Davis and Briggs ('48c) have reported that a growth effect similar to that produced by cellulose is obtained with levulinic acid. In another report, Hill and Briggs ('50) mention a growth effect from levulinic acid or xylose. Almquist et al. ('40) and Stokstad et al. ('41) have reported growth stimulation with glucuronic acid, calcium gluconate, and certain pentoses, among them xylose. In order to determine whether the growth promoting effect of cellu flour might be explained on some basis other than that presented above, two of these substances — calcium gluconate and levulinic acid — were studied: table 3 shows the results obtained with various combinations of levulinic acid, calcium gluconate and cellu flour at protein levels of 15 and 20%.

It is evident that neither calcium gluconate nor levulinic acid produced a growth effect with these diets. Cellu flour stimulated growth as a result of increased food consumption in every instance at the 15% protein level. No growth response was obtained from any of the supplements at the 20% protein level. There was, however, increased food consumption on all diets to which cellu flour was added.

DISCUSSION

The data presented here provide a simple basis for the explanation of the growth-stimulating effects observed when chicks are fed diets high in cellulose. The effects found by Davis and Briggs ('47) may possibly be explained on the basis of increased food consumption at a marginal level of protein. These authors, however, do not supply data on food consumption. Stimulation of growth by the addition to the purified diet of either cellu flour or extra protein (Lepp et al., '49) is quite simply explained on the basis of the results reported here.

It is generally recognized that analyses of food on the bases of crude protein, fat and fiber, are not reliable guides to nutritional value. Too little is known about the other chemical constituents. Thus the growth-depressing effect of alfalfa meal on chicks often attributed to its fiber content can be explained on the basis of a water-soluble constituent. Fiber or bulk in the diet does not depress either food intake or growth except at such high levels that the capacity of the animal is taxed. The idea that "locking up" of nutrients by fibrous feedstuffs makes them less available is not involved here, since we are referring to diets that are nutritionally adequate, exclusive of the bulky portion of the diet.

The primary factor in the voluntary food intake of young chicks appears to be the need for energy. Dietary protein influences food intake through its effect on body size. Thus an animal on a low-protein diet does not increase its food intake to obtain more protein, but if the available energy level of the same diet is reduced the animal increases its food intake.

SUMMARY

Diets containing varying dietary levels of protein, cellu flour (wood-pulp cellulose) and glucose were employed to study their effects on the ad libitum food consumption and growth of 9-day-old chicks maintained for a 20-day experimental period. In one experiment, 4 protein levels (5.3, 10.7,

16.0 and 21.3%) were fed at each of 5 levels of cellu flour (0, 12, 24, 36 and 48%). Food consumption (and as a result of this, growth rate) was stimulated by replacing glucose with moderate amounts of cellu flour. Food intake increased to satisfy the energy needs; as a result, the protein intake also increased and more rapid growth resulted. At very high cellu flour levels the energy level was low and not enough food could be consumed to satisfy the need for energy.

At the lower levels of cellu flour the growth rate was determined by protein intake. Growth could be increased either by raising the protein level of the diet or by decreasing the density of calculated metabolizable energy in the food (i.e., by increasing the bulk), which by increasing the food consumption resulted in an increased protein intake. At higher levels of bulk, the volume of food consumed was high but the growth rate was limited by an energy deficiency caused by the low energy level of the diet.

Criteria of food utilization were also compared. With diets containing high levels of cellu flour, weight gain ÷ food consumed was not a satisfactory measure of food utilization. Estimation of nutrients consumed by subtraction of cellu flour content from food consumed allowed calculation of a ratio of gain in weight to energy consumed that was about constant for each protein level.

Low protein diets produced carcasses of high fat content. Addition of cellu flour to the diet decreased the fat content.

Levulinic acid and calcium gluconate, substances which have been observed by some investigators to stimulate growth, were without effect with these diets.

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LIPID DEFICIENCY IN THE CALF [1, 2]

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TWO FIGURES

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It has been demonstrated that removal of fat from the diet of the rat will cause fat deficiency symptoms and that the syndrome can be alleviated by appropriate dietary supplementation. The essential fatty acids for the rat are thought to be one or more of the following: linoleic, linolenic and arachidonic (Turpeinen, '38; Rieckehoff et al., '49). The latter workers demonstrated the conversion of linoleic and linolenic acids to arachidonic acid in the rat. Greenberg et al. ('51) demonstrated that methyl arachidonate has 3.5 times the biopotency of linoleic acid when fed to fat-deficient rats.

Essential fatty acid deficiency symptoms in the rat have been described by Burr and Burr ('29, '30). Lipid deficiency syndromes also have been produced in the pig (Witz and Beeson, '51), mouse (White et al., '43), chick (Reiser, '50) and in certain insects (Fraenkel and Blewett, '46). The present investigation was designed to evaluate the role of lipids in the nutrition of the young calf.

EXPERIMENTAL

Twenty young calves of the Holstein, Brown Swiss and Milking Shorthorn breeds were obtained from the Iowa State

[1] Journal Paper J-2379 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 814.

[2] Supported in part through funds provided by the Western Condensing Co., Appleton, Wis.

College dairy herd during 1951–52. The first two calves on the experiment, 3596 and 3597, were allowed to remain with their respective dams for three days following birth. Calves 3603, 3640, 3643 and 3688 were taken from their dams at birth and thus did not consume colostrum. Subsequent calves were allowed one nursing of colostrum from their respective dams. The calves were placed in individual pens in a barn

TABLE 1

Composition of the semi-synthetic milk

COMPONENTS	AMOUNT
	%
Casein (vitamin-test)[1]	3.5
Fat	0–3.0
Salts [2]	0.2
Lecithin	Variable
Lactose, c.p.[3]	5.0
Vitamins [4]	. .

[1] Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. (Chemical analyses indicated the casein to be essentially lipid-free.)

[2] Mineral mixture as described by Kastelic et al. ('50).

[3] Chemical analyses indicated the lactose to be essentially lipid-free.

[4] Vitamins were fed in the amounts prescribed by Kastelic et al. ('50) with the addition of 1.9 μg vitamin B_{12} per calf daily.

which during the winter months was maintained at a temperature of about 60°F. by a thermostatically-controlled oil furnace.

It was observed that the calves consumed some wood shavings (bedding) during the latter part of their experimental period; therefore the last 5 calves, 3768, 3773, 3780, 3784 and 3786, were muzzled throughout the experiment.

The calves were fed a semi-synthetic milk (table 1) which in some cases contained various lipids. In addition, each calf received 80 mg of crystalline aureomycin hydrochloride per day. The amount of milk fed was reduced by one-half at the feeding immediately following the detection of diarrhea

and was increased gradually when the scouring subsided. In severe cases of diarrhea Kaopectate [3] or aureomycin, or both, were administered at therapeutic levels with warm water.

The semi-synthetic milk was prepared as described by Clark ('27) except that 0.1 N HCl was substituted for 0.1 N H_2SO_4 in the final adjustment of the pH to 6.6 (a modification suggested by Kastelic et al., '50). The milk then was transferred to 10-gallon milk cans and was stored in a cooler maintained at 40°F. until time for feeding. The milk was prepared in 20- to 30-gallon lots at two- to three-day intervals, depending upon the number of calves in the experiment.

Just prior to feeding, the milk was warmed to about 100°F. and vitamins were added. When lipids were used, they were added to the milk and the mixture was homogenized at approximately 2,500 pounds' pressure.

Rations fed to the various calves are indicated in table 2. All calves in group I received fat-free semi-synthetic milk for 8 weeks. The milk intake of 5 of the calves was subsequently reduced and lipids were added in an attempt to obtain recovery. Three other calves which appeared to be in fairly good health at 8 weeks of age were fed the semi-synthetic milk for an additional 4 weeks before lipids were introduced. Calves in the other groups received various lipids throughout the experimental period.

The diets fed to calves in groups I and II were isocaloric. Calves in groups III and IV received a higher caloric intake to enable observation of the effect of added calories upon gain in weight.

Body weights and clinical observations of the animals were recorded daily. At weekly intervals a blood sample was obtained from the jugular vein of each calf approximately three hours after feeding. (Heparin was used as the anticoagulant.) The plasma fat values were determined by the procedure developed by Allen ('38). Another portion of the plasma was used for the microdetermination of phospholipids as

[3] Produced by the Upjohn Company, Kalamazoo, Mich.

TABLE 2

Dietary regimes for calves in the various experimental groups

GROUP	CALF NO.	BREED [1] AND SEX	WEEKS ON EXPERIMENT 0–8 SSM, % b.w. daily [2]	0–8 Lipid added, % [3]	8–12 SSM, % b.w. daily [2]	8–12 Lipid added, % [3]	Over 12 SSM, % b.w. daily [2]	Over 12 Lipid added, % [3]
I	3596	H–F	16		11	H–2.0	12	B–2.0
	3597	H–F	16		16		11	B–2.0
	3603	H–M	16		13	C–1.5	11	C–1.5,L–0.5
	3699	H–M	16		16		11	C–2.0
	3700	H–M	16		16		11	H–1.8,L–0.2
	3768	MS–M	16		16	L–0.2	13	H–2.8,L–0.2
	3773	H–M	16		16	ME–5 gm	16	ME–15 gm
	3780	H–M	16		16	PLF–15 gm		
II	3657	BS–M	11	H–1.8,L–0.2				
	3643	BS–M	11	H–1.8,L–0.2				
	3771	MS–M	11	H–1.8,L–0.2	11	H–1.8,L–0.2		
III-A	3686	BS–M	11	H–3.0	11	H–3.0		
	3698	H–M	11	H–3.0	11	H–3.0		
III-B	3701	H–M	11	H–2.8,L–0.2	11	H–2.8,L–0.2		
IV-A	3696	MS–M	13	H–3.0	13	H–3.0		
IV-B	3697	H–M	13	H–2.8,L–0.2	13	H–2.8,L–0.2		
IV-C	3688	MS–M	13	B–3.0	13	B–3		
V-A	3640	BS–M	16	L–0.2				
V-B	3786	H–M	16	FFA–15 gm	11	H–1.5,FFA–15 gm		
V-C	3784	H–M	16	FFA–15 gm	11	FFA–15 gm		

[1] BS–Brown Swiss, H–Holstein, MS–Milking Shorthorn.

[2] SSM = Semi-synthetic milk; per cent of body weight daily.

[3] Per cent of milk, unless otherwise indicated: H = hydrogenated soybean oil, C = crude soybean oil, B = butter oil, L = lecithin, ME = methyl esters (approximately 50% oleate and 50% linoleate), FFA = free fatty acids (Emersol 9305), PLF = pork liver fat.

described by Zilversmit and Davis ('50). Total fatty acids were separated by the method of Wilson and Hansen ('35) and were determined by the micro-oxidative technique of Boyd ('38). Finally, aliquots of total fatty acids were taken for estimation of the plasma polyunsaturated fatty acids following alkali isomerization, which was accomplished by heating the fatty acids with potassium hydroxide-ethylene glycol reagent for 30 minutes at 180°C. under an atmosphere of nitrogen. The potassium hydroxide-ethylene glycol reagent was prepared by a modification of the method of O'Connor et al. ('45). A Beckman spectrophotometer, Model DU, was employed to measure optical densities at wave lengths recommended by Brice and Swain ('45) for estimating linoleic, linolenic and arachidonic acids. Calculations of the polyunsaturated fatty acids were based upon the equations developed by the latter workers.

RESULTS

Body weight changes

The average weekly body weight changes for calves in groups I, II, IIIA and IV are indicated in figure 1. Calves in group I did not lose so much weight initially as calves that received lipids. Subsequently, however, calves on the lipid-free milk gained weight at a very slow rate, whereas calves receiving lipids began to grow at a comparatively rapid rate at about two weeks of age. The mean weight gain from zero to 56 days was 10.9 pounds for calves fed the lipid-free milk, as compared to 30.3 pounds for those (group II) that received an isocaloric diet which contained 1.8% hydrogenated soybean oil and 0.2% lecithin.

Three of the more vigorous calves at 8 weeks (group I) were fed the lipid-free milk for an additional 4 weeks. During the latter period these animals gained an average of 8.3 pounds, whereas the one remaining calf in group II, on an isocaloric diet, gained 30.5 pounds during the same period. After the 12-week depletion period various lipids were intro-

duced into the diets of these three calves (group I) and recovery resulted.

Other external manifestations of lipid deficiency

After 6 weeks on the lipid-free diet, other deficiency symptoms began to appear. At 8 weeks the syndrome was quite severe in about 50% of the animals. Scaly dandruff appeared over many areas of the body but was most pronounced on the shoulders, back and tail. The hair was long and dry and

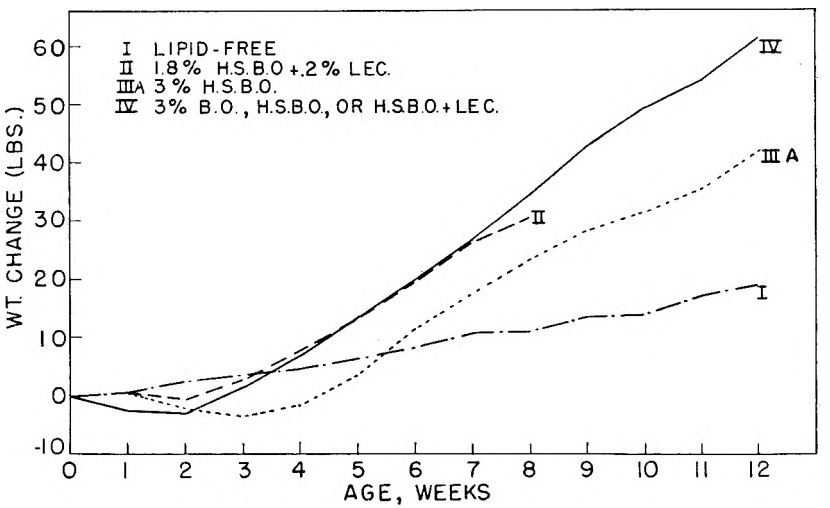

Fig. 1 Effect of dietary on changes in group mean weights of calves.

lacked the luster of the hair on calves receiving fat. Alopecia was occasionally observed on the neck and tail of calves fed the lipid-free diet.

The incidence of diarrhea in all groups was comparatively high during the second week. Except for group V, however, the incidence of diarrhea was low from the second to the 8th weeks in all groups receiving lipid supplementation. The incidence of diarrhea increased sharply at about 8 weeks among all calves receiving lipid-free milk, but remained low among those calves receiving lipid supplementation. Two

of the calves in group V, 3784 and 3786, received about 0.2% free fatty acids, while calf 3640 received lecithin in the semi-synthetic milk. These lipids fed singly in the synthetic milk seemed to increase the incidence of diarrhea, whereas when they were fed in combination with fats (other than crude soybean oil), diarrhea was not excessive.

After 8 weeks on the experiment, 5 calves (group I) were given various lipids, replacing lactose and casein to maintain an isocaloric intake per 100 pounds of body weight. The three other calves in group I received similar supplements after 12 weeks. Subsequently, the calves became more alert, the dandruff condition was alleviated, the skin became more oily and a smooth haircoat developed, the loss of hair ceased and new hair began to grow in areas which previously showed alopecia. Butter oil, methyl esters of oleic and linoleic acids, and hydrogenated soybean oil plus lecithin were most effective in relieving the deficiency symptoms. Hydrogenated soybean oil alone effected partial recovery. Crude soybean oil seemed to promote some improvement, but the effects were obscured by an increase in diarrhea, which was probably due to the oil or some component therein. Pork liver residue (20% fat) effected recovery when fed at a level which supplied 15 gm fat per 100 pounds' body weight daily. This product contained relatively large amounts of linoleic and arachidonic acids. Moreover, the lecithin and free fatty acid levels were high. Thus, although this product promoted recovery, it was not particularly useful in ascertaining the specific lipid (or lipids) required.

Blood plasma lipid levels

The effect of several dietaries on the mean values of certain blood plasma lipids is shown in figure 2. After the first week, animals fed only the fat-free semi-synthetic milk (group I) exhibited low plasma fat values, whereas animals (group II) that received 1.8% hydrogenated soybean oil plus 0.2% lecithin had the highest blood plasma fat values. Calves in group

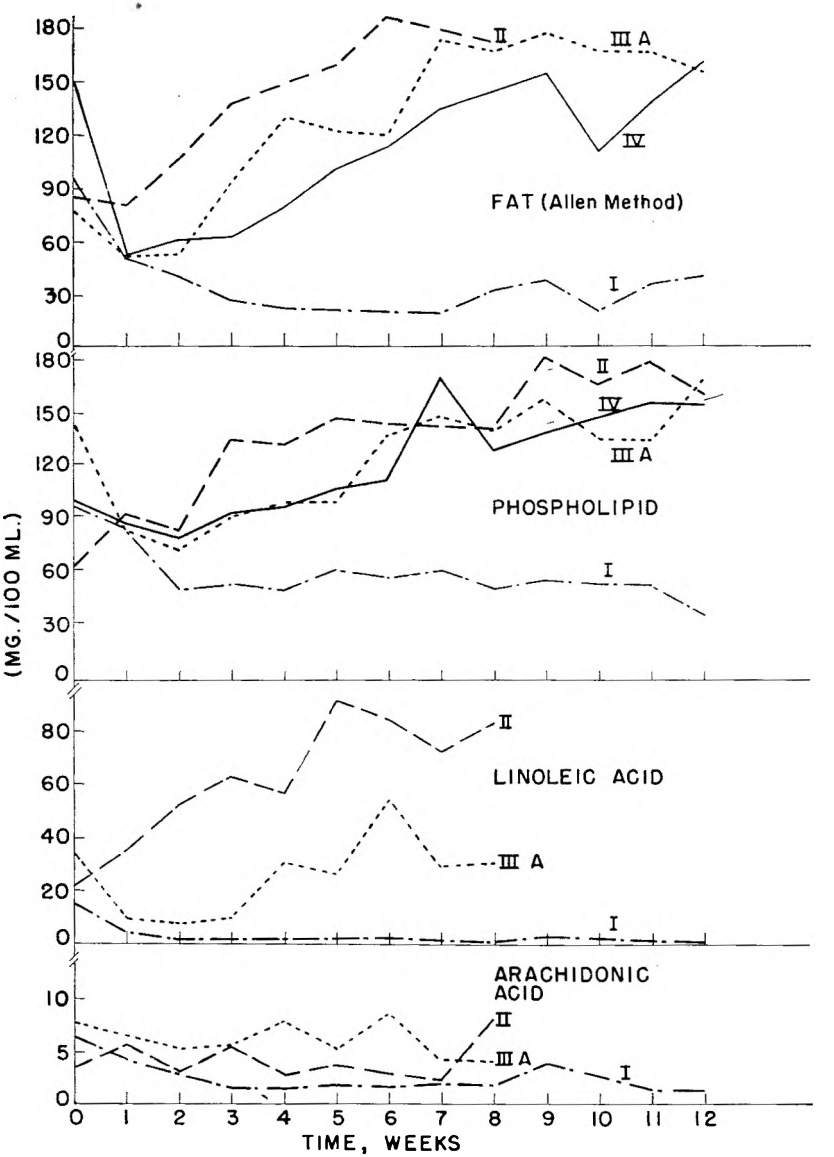

Fig. 2 Effect of dietary on mean values of certain blood plasma lipids. (I = lipid-free; II = 1.8% hydrogenated soybean oil + 0.2% lecithin; IIIA = 3% hydrogenated soybean oil; IV = 3% butter oil, hydrogenated soybean oil, or hydrogenated soybean oil + lecithin.)

IIIA receiving hydrogenated soybean oil at a 3% level had lower plasma fat values than those in group II despite the lower lipid intake (2%) of the latter.

In most instances the plasma phospholipid and polyunsaturated fatty acid values were markedly lower for calves fed the lipid-free diet than for those receiving lipids. Although the arachidonic acid levels were lowest for calves in group I, the effect of diet on this fatty acid was less marked than that noted for linoleic acid.

The linolenic acid values were low (less than 5 mg/100 ml of plasma) and variable in all instances regardless of diet. Trends in the total plasma fatty acid values were similar to those indicated for "Allen fat." The plasma fatty acid levels for calves in group II remained consistently higher than the values for calves in the other groups.

DISCUSSION

The data presented herein indicate that dietary lipids are required both quantitatively and qualitatively by the young calf for normal growth and development. Growth retardation was apparent after the calves had received the lipid-free diet for approximately three weeks. Similar depression of growth has also been observed in rats, although the length of time required for exhibition of symptoms has been highly variable. Panos and Finerty ('53) found impairment of growth of rats on a fat-free ration within two weeks. Others (Evans and Lepkovsky, '32; Greenberg et al., '50) have reported that a plateau in rat growth occurs between 60 and 77 days. Burr and Burr ('29) and Turpeinen ('38), however, reported gain in weight of rats on a fat-free ration up to approximately 112 to 119 days.

At about 6 weeks of age calves receiving the lipid-free milk began exhibiting other deficiency symptoms, including dandruff, dull haircoat and partial alopecia. A similar condition has been observed in the rat after 9 to 12 weeks on a fat-deficient diet (Burr and Burr, '29; Panos and Finerty,

'53) and in the pig after a depletion period of 6 to 9 weeks (Witz and Beeson, '51). The rats and pigs received lipids until weaning time, thus possibly accounting for the comparatively long depletion periods. In the present study, lipids in most instances were excluded from the diet of the calves (in group I) after the first feeding post partum. Limited colostrum ingestion appeared to have no significant influence on the rate of development of the fat deficiency syndrome and was helpful in the prevention of the crisis period observed in calves fed no colostrum (Kastelic et al., '50).

In the present investigation the lipid deficiency syndrome was prevented or alleviated by (a) butter oil, (b) hydrogenated soybean oil plus lecithin, and (c) mixed methyl esters of oleic and linoleic acids, respectively. Thus, the essential lipid component or components apparently were present in each of the above lipid combinations. The observations made on the methyl esters were restricted to one calf and therefore conclusions based on these data should be tentative only. Nevertheless, the prompt recovery following administration of the methyl esters would suggest that oleic or linoleic acids, or both, may be primarily involved.

Samples of lipids used in the present study were analyzed for polyunsaturated fatty acids to enable calculation of the amounts of these components in the various diets. Greenberg et al. ('51) have demonstrated that methyl arachidonate has 3.5 times the biopotency of linoleic acid for alleviation of lipid deficiency in the rat. If this factor is applied to the present data, the calves fed butter oil received polyunsaturated fatty acids equivalent to 12.6 gm of linoleic acid per 100 pounds of body weight daily.

Calves did not respond readily when hydrogenated soybean oil, a good source of oleic acid but supplying only 0.7 gm polyunsaturated fatty acids per 100 pounds of body weight daily, was added to the diet after the lipid depletion period. Moreover, the weight gains of calves fed hydrogenated soybean oil as the only lipid from the beginning of the experimental period were subnormal. This suggests that if oleic

acid is essential in the dietary of the calf, other lipids (possibly linoleic acid) may be required for maximum response. Further support is given to this postulate by the favorable response of calves to hydrogenated soybean oil plus lecithin, which supplied about 4 gm of linoleic acid per 100 pounds of body weight daily. It is possible, however, that the beneficial effects derived from lecithin may be due in part to factors other than polyunsaturated fatty acids (Jones et al., '48; Kastelic et al., '50). The substitution of lecithin for 10% of the hydrogenated soybean oil resulted in marked increases in blood plasma fat values, but whether this change was directly associated with the improved growth and alleviation of lipid deficiency symptoms is not clear.

Linoleic acid levels in the blood plasma of calves fed the lipid-free diets were low, but this was also observed for most of the other blood plasma lipids that were measured. Thus, on the basis of the present data it is difficult to associate the level of any one of the blood plasma lipids with the deficiency syndrome. Unfortunately, the administration of free fatty acids and of lecithin was followed by marked increases in the incidence of diarrhea, which tended to obscure any ameliorating effects of the supplementary lipids.

Although this investigation has indicated the essentiality of lipids in the diet of the young calf, there is a need for further research concerning the role of the various lipids, particularly lecithin and the unsaturated fatty acids. Studies designed to clarify some of these relationships are now under way and will be reported later.

SUMMARY

The dietary essentiality of lipids for young dairy calves was studied by feeding a "lipid-free," semi-synthetic milk containing casein, lactose, minerals and vitamins. Responses to various lipid supplements were evaluated. Weights and clinical observations were recorded daily for each calf. Weekly blood plasma samples were analyzed for total fatty acids,

"Allen fat," phospholipids and for linoleic, linolenic and arachidonic acids.

Marked retardation of growth (weight gain) was observed after calves were fed the lipid-free diet for approximately three weeks. Other lipid deficiency symptoms which were quite severe in approximately 50% of the calves at 8 weeks included scaly dandruff; long, dry hair; excessive loss of hair on the back, shoulders and tail; and diarrhea.

Lipids which prevented development of, or promoted prompt recovery from, the deficiency syndrome were (a) butter oil, and (b) hydrogenated soybean oil plus lecithin. Limited data indicate that the methyl esters of fatty acids (approximately 50% oleic and 50% linoleic) also promote recovery. Response to hydrogenated soybean oil alone was not so great as when lecithin also was supplied. The mean weight gains from zero to 56 days for calves fed the lipid-free diet and for those fed an isocaloric diet containing hydrogenated soybean oil and lecithin were 10.9 and 30.3 pounds, respectively.

Blood plasma "Allen fat," total fatty acids, phospholipids and linoleic acid were significantly lower in the calves receiving the lipid-free milk than in calves receiving lipids. Differences among the various dietary groups in the blood plasma linolenic and arachidonic acid contents were small and the values for the former were low in all instances.

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EFFECTS OF SALTS ON THE INSTABILITY OF THIAMINE IN PURIFIED CHICK DIETS [1]

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Previous studies (Hankes and Elvehjem, '49; Lyman and Elvehjem, '51) have shown thiamine to be unstable in purified diets for rats, and this instability is at least as serious in certain purified diets for chicks (Waibel et al., '53). Glycerol stabilizes thiamine in both types of diets (Kandutsch and Baumann, '53; Waibel et al., '53). The present study deals with the effect of salt mixtures on the stability of thiamine, with certain effects of humidity, and with a comparison between thiamine chloride hydrochloride and thiamine mononitrate.

EXPERIMENTAL METHODS

The basal diet consisted, in per cent, of sucrose, 61; hot alcohol-extracted casein, 18; gelatin, 10; salts, 6; soybean oil, 4; feeding oil [3] (2,000 A–300 D), 0.5; DL-methionine, 0.3; choline chloride, 0.2; and inositol, 0.1 gm per 100 gm. The following vitamins were added, in milligrams per kilogram: biotin, 0.2; menadione, 0.5; alpha-tocopherol, 3.0; pyridoxine · HCl, 4.0; folic acid, 4.0; riboflavin, 6.0; calcium pantothenate, 20; niacin, 50; *p*-aminobenzoic acid, 100; and vitamin B_{12},

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[3] A commercial blend of fish oils and vegetable oils containing 2,000 U.S.P. units of vitamin A and 300 units of vitamin D per gram.

0.030. When glycerol was added, it replaced an equivalent amount of sucrose.

For stability comparisons between thiamine chloride hydrochloride and thiamine mononitrate, the diets were prepared in the following manner: choline chloride, DL-methionine and the vitamin mixture (including thiamine) were mixed into about half of the dietary sucrose (mixture A). The oils were added to the remaining sucrose, followed by casein, gelatin, salts and glycerol, and these components were mixed together (mixture B). Mixtures A and B were then combined. For diets dealing with the effects of salts, mixtures A and B (less salts) were combined, and substances such as glycerol or an ascorbic acid premix were added to a depression made in the mixture, covered over, and the salts added on top. The diets were usually mixed in a Hobart feed mixer (model-301), and unless specified otherwise the diets were stored in nearly full 100 to 200-ml brown glass bottles with loose plastic covers.

The preparation of the diets used in the humidity experiments was as follows: mixtures A and B minus the oils were prepared as usual except that the sucrose was previously dried. The premixes were kept in desiccators over $CaCl_2$ until a relatively dry day arrived, dew point, 55°. Oils (not dried) and variables were added to aliquots of the premixes, and the diet was mixed by hand and placed immediately into beakers in appropriate desiccators. The diets in series 2, table 5, were prepared as were those involving variations in salt except that the sucrose was previously dried. Known humidities were achieved by means of $CaCl_2$, constant-humidity-H_2SO_4 solutions, or distilled water placed in the bottom of conventional laboratory desiccators. Under the humid conditions the diets absorbed sufficient moisture to affect the weight of the sample. Analyses for thiamine were made on the moist samples and the results corrected for the weight of the water absorbed.

All diets were analyzed for thiamine immediately after being mixed and at intervals thereafter by the thiochrome

method as recommended by the Association of Vitamin Chemists ('51), except that the enzymatic digestion step was omitted. Equimolar quantities of thiamine mononitrate and thiamine chloride hydrochloride yielded equivalent fluorescence in this determination. In certain preliminary studies the recoveries of thiamine were variable and seldom over 60% for diets of low thiamine content, in which 15 gm of diet supplied a total amount of 15 μg of thiamine per sample. When the salt mixture was omitted from the diet, the recovery

TABLE 1

Effect of fineness of salts V (Briggs et al., '43) on the destruction of thiamine chloride hydrochloride in purified chick diets [1]

SALTS IN DIET	THIAMINE REMAINING			
	Days after mixing diet			
	0	3	10	28
	%	%	%	%
None (sucrose)	99	96	82	58
6% Salts V, mixed in mixer	92	51	27	..
6% Salts V, ball-milled 1 hour	95	21	..	..
6% Salts V, ball-milled 3 hours	101	18	..	..

[1] Diets contained 3.0 μg of $B_1Cl \cdot HCl$ per gram and were kept at 37°C. and 55% relative humidity.

of thiamine was complete. For diets containing salts, an increase in the amount of Decalso in the ion exchange column from about 1.0 gm to 2.5 gm resulted in complete recoveries. Apparently cations of the salt mixture compete with thiamine for position on the exchanger, for the original level of Decalso had given satisfactory recoveries with purified diets for rats. These latter contained only 4% salts as compared with 6% salts in the chick diets.

RESULTS

Fineness of salts V (Briggs et al., '43)

In agreement with a previous study (Kandutsch and Baumann, '53), thiamine chloride hydrochloride in diets contain-

ing salts was much less stable than in a salt-free ration (table 1). Furthermore, an increase in the fineness of salts by ball-milling them for one or three hours markedly hastened the destruction of thiamine. The harmful effect of very fine salts has also been seen in several other studies (Waibel, '53). Thiamine chloride was relatively stable when sucrose replaced salts V in the diet (table 2, diet 1), although some destruction did occur.

TABLE 2

Effect of certain inorganic salts on the stability of thiamine chloride hydrochloride in purified chick diets

SALTS IN DIET [1]	THIAMINE REMAINING [2]							
	Days after mixing							
	Series 1			Series 2				
	37°C.			37°C.			24°C.	
	0	3	10	0	3	10	7	19
	%	%	%	%	%	%	%	%
1. None	92	93	73	97	89	55	92	84
2. $CaCO_3$, K_2HPO_4, $CaHPO_4 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, NaCl	76	17	..					
3. Ferric citrate	92	95	73					
4. KI	99	94	64					
5. $MnSO_4 \cdot H_2O$	96	86	21					
6. $ZnCl_2$	94	95	78					
7. $CuSO_4 \cdot 5H_2O$	95	86	50					
8. Salts V	87	19	..	97	20	..	24	12
9. $ZnCl_2$, $CuSO_4 \cdot 5H_2O$, KI, Fe citrate, $MnSO_4 \cdot H_2O$				100	75	18	86	21
10. $CaCO_3$				101	73	46	80	53
11. $CaHPO_4 \cdot 2H_2O$				96	91	65	90	80
12. K_2HPO_4				83	22	..	32	21
13. $MgSO_4 \cdot 7H_2O$				97	86	65	92	83
14. NaCl				94	92	77	97	91
15. $Ca_3(PO_4)_2$ + KCl to supply at least as much Ca, P and K as in diets 10, 11 + 12				95	99	89	97	90
16. As 15, + $MgSO_4 \cdot 7H_2O$, NaCl, $ZnCl_2$, $CuSO_4 \cdot 5H_2O$, KI, Fe citrate, and $MnSO_4 \cdot H_2O$				95	94	81	92	90

[1] The amounts of the various salts were those equivalent to 6% of salts V (Briggs et al., '43); they were made up to 6% with sucrose, and ball-milled for one and one-half hours.

[2] Three micrograms of $B_1Cl \cdot HCl$ per gram of all diets at start of experiment.

Components of salts V

The most destructive component in salts V appeared to be K_2HPO_4 (table 2, diet 12). $CaCO_3$ (diet 10) also hastened the destruction of thiamine somewhat, while $MnSO_4 \cdot H_2O$ produced a delayed effect (diet 5, and probably 9). The other components of the salt mixture did not appear to be seriously implicated in the destruction of thiamine during these short time experiments. Thiamine was relatively stable when the Ca, P and K of the older mixture were furnished by $Ca_3(PO_4)_2$ and KCl (table 2, diet 15), and these latter salts were also innocuous in the presence of the other components of the salt mixture (diet 16). The latter mixture, A, was incorporated into the diets at levels of 3 to 8% and compared with salts V for thiamine stability and chick growth. After one week in the chick room the diets showed decreasing amounts of thiamine as the amount of salts V was increased, only 27% remaining in the diet containing 8% of salts V. On the other hand, the diets containing salts A retained 94 to 96% of the thiamine. However, growth on salts A was not always equal to that on salts V (Waibel, '53) and hence further modifications in the mixture will still have to be made. There is also the possibility that salts favorable to thiamine may be unfavorable to some other nutrient (Frost, '43).

Salts of thiamine

Hollenbeck and Obermeyer ('52) have reported thiamine mononitrate to be somewhat more stable in fortified flour than thiamine chloride hydrochloride. Their experiments were conducted under relatively mild conditions, so that 60% of the thiamine chloride remained after one year. A series of comparisons at controlled temperatures, 4°C., 27°C. and 37°C., under our more drastic conditions (Waibel, '53) failed to reveal any consistent difference in lability between the two salts of thiamine, except for two series in which appropriate diets were fed to chicks and parallel chemical analyses for thiamine were made (table 3). Both the survival and the

TABLE 3

Comparison of the stability of thiamine chloride hydrochloride and thiamine mononitrate in diets and of their biological activity for chicks

DIET	AVERAGE CHICK WEIGHT [1]			PER CENT THIAMINE REMAINING [2]		
	Expt. 1		Expt. 2	Diets in animal room [3]		Diets in constant temp. chamber
	1 wk.	2 wk.	2 wk.	1st wk.	2nd wk.	
	gm	*gm*	*gm*	%	%	%
				Expt. 1		Expt. 1 3 days at 37°C.
1.20 μg $B_1Cl \cdot HCl$/gm	56	72 (11)		49	35	52
1.20 μg $B_1Cl \cdot HCl$/gm + 1% glycerol	64	122 (15)		89		77
1.164 μg B_1NO_3/gm	66	96 (14)		68	49	56
1.164 μg B_1NO_3/gm + 1% glycerol	64	122 (13)		88		74
				Expt. 2		Expt. 2 7 days at 24°C.
6.0 μg $B_1Cl \cdot HCl$/gm	68	138 (15)	142 (24)	50		58
5.822 μg B_1NO_3/gm	65	126 (13)	139 (25)	63		64

[1] Numbers in parentheses indicate survivors at 14 days. Fifteen chicks per group in experiment 1; 25 in experiment 2.

[2] The diets of experiment 1 were those prepared at the beginning of the second week of the feeding trial. The diets of experiment 2 were those prepared at the beginning of the feeding trial. Diets were kept in brown glass bottles with loose lids. Relative humidity, 55% in experiment 1; 65% in experiment 2.

[3] Feed for analysis was taken from feeders.

weight of the chicks on the nitrate were somewhat better than when the chloride was used in an unstabilized ration, although in the presence of glycerol this difference disappeared. Chemical analyses tended to parallel the biological results. Glycerol proved to be an effective stabilizer for both

TABLE 4

Effect of controlled humidity on thiamine destruction [1]

DIET	THIAMINE REMAINING after 14 days at 24°C. % Relative humidity			
	0	10	90	100
	%	%	%	%
Series 1 (Regular salts V)				
1. $B_1Cl \cdot HCl$	28	29	63	
2. $B_1Cl \cdot HCl$ + 500 p.p.m. ascorbic acid	40	42	50	
3. $B_1Cl \cdot HCl$ + 1% glycerol	62	64	83	
4. B_1NO_3	34	36	62	
5. B_1NO_3 + 500 p.p.m. ascorbic acid	46	51	48	
6. B_1NO_3 + 1% glycerol	61	68	80	
Series 2 (Thiamine Cl · HCl. Substitutions for salts V, all ball-milled)				
7. Sucrose	93			95
8. Salts A (table 4)	95			89
9. Salts V	14			62
10. Salts V + 500 p.p.m. ascorbic acid	17			52
11. Salts V + 1% glycerol	36			78
12. K_2HPO_4	28			69
13. K_2HPO_4 + 500 p.p.m. ascorbic acid	36			60
14. K_2HPO_4 + 1% glycerol	59			79
15. KH_2PO_4	90			91
16. $CaCO_3$	84			90
17. $CaCO_3$ + 500 p.p.m. ascorbic acid	93			72
18. $CoCO_3$ + 1% glycerol	95			95
19. $MnSO_4 \cdot H_2O$	86			90
20. $MnSO_4 \cdot H_2O$ + 500 p.p.m. ascorbic acid	89			82
21. $MnSO_4 \cdot H_2O$ + 1% glycerol	92			92

[1] All diets contained the equivalent of 3.0 μg of $B_1Cl \cdot HCl$/gm and were kept in desiccators. They were analyzed for thiamine when prepared and gave good recoveries. They were also analyzed at 7 days, giving trends in all cases suggestive of the results reported here.

salts of thiamine, and at the relatively high level of thiamine equivalent to 6.0 μg of the hydrochloride per gram of diet, both salts were equally active for the growth of chicks.

Effect of humidity

The destruction of both thiamine chloride hydrochloride and thiamine nitrate appeared to be retarded by the presence of moisture (table 4, diets 1, 4 and 9), and the addition of 1% of glycerol was more protective in the dry environment than the moist atmosphere (diets 1 and 3, 4 and 6). In contrast to the stabilizing effect of ascorbic acid on thiamine in rat diets (Kandutsch and Baumann, '53), ascorbic acid was ineffective in chick diets at moderate humidities (Waibel et al., '53); in the present experiments ascorbic acid appeared to have a slight stabilizing effect on thiamine in the dry atmospheres but it hastened the destruction of thiamine in the moist diets (table 4, diets 1, 2; 4, 5; 9, 10; 12, 13; 16, 17; and 19, 20).

DISCUSSION

A number of published reports are consistent with the conclusion that the ingredient in salts V which is most destructive to thiamine is K_2HPO_4. Using a somewhat different technique (growth response on the addition of single vitamins to a deteriorated diet), Rombouts ('53) concluded that it was the salt mixture which was mainly responsible for the instability of thiamine and other vitamins. In these studies the mixture of Phillips and Hart ('35), which contains K_2HPO_4, was particularly deleterious, whereas the U.S.P. ('36) mixture, containing no added K_2HPO_4, was relatively innocuous. Reyniers et al. ('50) observed thiamine to be stable at room temperature in a purified ration with salt mixture 12 of Jones and Foster ('42), which contains no K_2HPO_4. Lyman and Elvehjem ('51) found thiamine to be unstable in purified diets that contained salts IV of Hegsted et al. ('41), another mixture containing K_2HPO_4. A number of possible reasons for the destructiveness of this particular

salt can be suggested, since it is highly alkaline, highly soluble and very hygroscopic. The related salt, Na_2HPO_4, which is non-destructive to thiamine, is as alkaline as the potassium salt but it is less soluble (12 vs. 33 parts per 100 parts of water; Lange, '46) and it is also less hygroscopic. When 5-gm portions of the two salts were spread on a watch glass and allowed to stand for 45 minutes, the weight of the Na_2HPO_4 remained the same, while the K_2HPO_4 gained 0.108 gm. After 20 hours in the laboratory the Na salt was still a powder, while the K salt had turned into a partially cloudy puddle.

Presumably it is the combination of moisture and K_2HPO_4 that is so deleterious to thiamine, with the vitamin dissolving in the concentrated alkaline solution that forms about the ration particles as the salt absorbs moisture from the air. The fact that the vitamin was often more stable in a very moist atmosphere (soggy diet) than at lower humidities (table 4) may have been the result of further dilution. The rather consistent effect of glycerol in stabilizing thiamine may also be ascribed to its role as a humectant. On the other hand, Hollenbeck and Obermeyer ('52) observed the stability of thiamine in enriched flour to decrease with an increase in moisture, while Kandutsch and Baumann ('53) found the stability of thiamine in a rat diet to be improved in a dry atmosphere. It would therefore appear that the role of moisture in the preservation or destruction of thiamine still merits further study.

SUMMARY

1. Thiamine was particularly unstable in purified chick diets containing salts V. The ingredient most responsible for this destruction was K_2HPO_4; $CaCO_3$ and $MnSO_4$ also contributed to the instability of thiamine. The stability of thiamine was decreased further when the salt mixture was ball-milled.

2. A new salt mixture, devoid of the two most deleterious ingredients of salts V, permitted thiamine stability under the conditions tested.

3. Thiamine mononitrate was somewhat more stable than thiamine chloride hydrochloride under certain conditions, although when the destruction of thiamine was very rapid, its use was of less importance than the composition of the salt mixture.

4. One per cent of glycerol reduced the disappearance of thiamine under a wide variety of conditions. On the other hand, ascorbic acid was protective only under relatively dry conditions, and in a moist environment it sometimes hastened thiamine destruction. The stability of thiamine varied widely with humidity.

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THE MATERNAL DIET AS A SOURCE OF GROWTH FACTORS TRANSMITTED BY THE HEN THROUGH THE EGG TO THE PROGENY [1]

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INTRODUCTION

The favorable influence on the growth of the progeny when casein is incorporated in the maternal diet has been reported previously by Csonka and Olsen ('49). Casein has been shown to be associated with a growth factor presumably identical with vitamin B_{12} (Cary et al., '46) and it has also been demonstrated that vitamin B_{12} present in the hen's diet is transmitted to the egg (Yacowitz et al., '52). Some additional factor in casein is believed by us to be involved, however, which is also transmitted through the egg to the progeny.

In the present report we show the growth behavior of chicks fed vitamin B_{12} or dried cow manure as a supplement to their basal diet and how differently they react to these supplements, depending on the maternal dietary regimen and its consumption for a short or an extended period by the laying hen.

Furthermore, it will be shown that vitamin B_{12} concentrate fed to the hen or its progeny does not necessarily accelerate chick growth; under certain dietary combinations it may

[1] This is the 4th article in a series dealing with the influence of dietary protein.

even act in a growth-inhibiting fashion. Information was also obtained on the relative efficacy of vitamin B_{12} concentrate when used as a supplement in the maternal diet or in the chick diet.

EXPERIMENTAL

Rhode Island Red pullets and roosters were placed in 4 pens; each pen held 30 pullets and three roosters.[2] The pullets and roosters in each pen were fed a specially prepared diet: in pen 1 the hens received a low-protein-corn diet[3] in which the protein came mainly from the corn; the feed in pen 2 was the same as that in No. 1 plus 0.2% B_{12} concentrate[4]; the hens in pen 3 were fed a high-protein-casein diet[5]; and in pen 4, the feed was the same as in pen 3 with a 0.2% B_{12} concentrate supplement. Two experimental conditions were investigated. In the first, hens were placed on the 4 experimental diets on January 16, 1951, approximately 90 days before egg collection commenced. Three replications were carried out, using the chicks hatched on May 5, May 29 and June 12, 1951. In the second, the diets of the hens were reversed on May 22, 1951, with respect to dietary proteins but not with respect to vitamin B_{12} supplementation. Egg collection was started two weeks later and three replications were conducted with chicks hatched July 10, July 21 and August 7, 1951.

The eggs were collected daily by trap-nesting, and were marked and dated for identification and placed in a cooler at 55°F. Once every two weeks all the eggs were placed in forced-draft incubators that maintained a temperature of 100°F. and a relative humidity of 55%. At hatching time each chick was wing-banded. The chicks were caged in electrically heated battery brooders with wire flooring to prevent

[2] We recognize that the pen arrangement did not prevent coprophagy among the birds.

[3] The composition of this diet was given in a previous article in this Journal (Csonka et al., *39:* 485, 1949).

[4] Merck.

[5] See footnote 3.

access to fecal materials. The chicks were weighed at hatching time and 14 and 27 days later. The chicks were killed and their sex determined at the end of the experiments.[6] Fourteen-day gains were measured for checking purposes and are not included in the discussion.

The following 4 chick diets were fed: (1) growing mash 1096 which was practically free of vitamin B_{12}, (2) chick mash 1096 with 0.2% B_{12} concentrate, (3) chick mash 1096 with

TABLE 1

Composition of chick mash "1096"

INGREDIENTS	AMOUNT
	%
Yellow corn	38.0
Barley	20.0
Alfalfa leaf meal	3.0
Soybean meal	35.0
B-Y feed (500 μg riboflavin per gram)	0.6
Bone meal	1.5
Limestone flour	1.0
Manganized salt (96% NaCl: 4% $MnSO_4$)	0.7
Feeding oil (1,200 I.U. of vitamin A and 400 A.O.A.C. units of vitamin D)	0.2
Niacin	1 mg/100 gm

5% dried cow manure,[7] and (4) chick mash supplemented with both B_{12} and cow manure.

Instead of basing our findings on one experiment with a large number of chicks, we preferred to carry out three replications. For each replication, each diet was fed generally to 40 randomly chosen chicks (10 originating from hens on each of the 4 maternal diets). The gains in body weight, representing growth, were expressed as "individual" means by averaging for each sex separately the gains of all the

[6] Male birds generally grow bigger; and so increase in weight, without knowing the sex distribution in the group, might lead to false conclusions.

[7] The fresh cow manure was collected in the months from May through July, 1951, from the same group of animals and was dried weekly at 80°.

chicks on the same hen-chick-dietary regimen. "Average" means over sex and "over-all" means over diet were found by combining the individual means. The vitamin B_{12} assay of the eggs and feed was made by microbiological analysis (Denton et al., '52). A composite sample of the yolks used for the test was taken from eggs collected in a separate experiment and laid by 5 hens during their third weekly feeding period.

RESULTS AND DISCUSSION

Analysis of variance was applied separately to the weight gains observed under the two maternal dietary conditions (series I, extended feeding, and series II, reversal) following the methods of Snedecor ('46) and Cochran and Cox ('50). Because of significant interactions involving dietary factors, the discussion will be based in general on the average means over sex rather than on the over-all means of the various diets. Since the analysis of variance for series I also indicated some evidence of sex versus maternal diet interactions, reference will be made to the individual means for each sex whenever the sexes appeared to respond differently. To compensate for the bias associated with unequal numbers of chicks per mean, all average and over-all means have been shown on the unweighted basis.

Standard errors for the individual means in the tables were based on the pooled error variance, while those for the average and over-all means were computed from the additive properties of variances. The error variance satisfied tests of homogeneity over the experiments.

Tests of significance for the two series (tables 2 and 3) were made by comparing the difference between two means with one and one-half times the sum of the corresponding standard errors. For the variation in numbers of chicks encountered in these experiments, such tests give a conservative approximation to the "t" test. In making these tests the limitations of the "t" test summarized by Duncan ('52) were kept in mind.

TABLE 2

The influence of the maternal diet (fed for an extended period) on chick growth with a variety of supplements[1] in both maternal and chick diet

EXP. NO.	HEN DIET	SEX OF CHICK	MEAN WEIGHT GAIN (NO. OF CHICKS) AND STANDARD ERROR				UNWEIGHTED OVER-ALL MEAN
			Chick mash 1096 without or with supplements				
			None	0.2% B_{12} conc.	5% Dried cow manure	B_{12} and cow manure	
1	Low-protein corn	♂	135 (13) 10	137 (11) 11	182 (13) 10	157 (11) 11	153 (48) 5
		♀	98 (9) 13	137 (13) 10	177 (11) 11	144 (11) 11	139 (44) 6
		average	116 (22) 8	137 (24) 8	180 (24) 8	150 (22) 8	146 (92) 4 [2]
2	Low-protein corn + 0.2% B_{12} conc.	♂	181 (14) 10	167 (16) 10	196 (14) 10	163 (12) 11	177 (56) 5
		♀	166 (15) 10	144 (13) 10	165 (16) 10	165 (18) 9	160 (62) 5
		average	174 (29) 7	156 (29) 7	180 (30) 7	164 (30) 7	168 (118) 3
3	High-protein casein	♂	164 (14) 10	156 (12) 11	204 (21) 8	175 (16) 10	175 (63) 5
		♀	193 (12) 11	171 (17) 9	194 (6) 16	172 (11) 11	182 (46) 6
		average	178 (26) 7	164 (29) 7	199 (27) 9	174 (27) 7	179 (109) 4
4	High-protein casein + 0.2% B_{12} conc.	♂	200 (13) 10	178 (12) 11	208 (14) 10	163 (17) 9	187 (56) 5
		♀	168 (15) 10	165 (17) 9	185 (14) 10	159 (12) 11	169 (58) 5
		average	184 (28) 7	172 (29) 7	196 (28) 7	161 (29) 7	178 (114) 4
Unweighted over-all mean		♂	170 (54) 5	160 (51) 5	198 (62) 5	164 (56) 5	173 (223) 3
		♀	156 (51) 5	154 (60) 5	180 (47) 6	160 (52) 5	162 (210) 3
		average	163 (105) 4	157 (111) 4	189 (109) 4 [3]	162 (108) 4	168 (433) 2 [4]

[1] The B_{12} concentrate (Merck) contains an equivalent of 12.5 mg vitamin B_{12} per pound and thus supplementation with 0.2% of this concentrate adds 5.52 μg vitamin B_{12} per 100 gm of feed.

[2] This figure is significantly less than the other three figures in this column.

[3] This figure is significantly greater than the other three figures in this line.

[4] Started with 460 chicks, of which 17 died and 10 lost their identification wing bands.

Series I. Maternal diets consumed for an extended period

The data for the first experimental conditions are summarized in table 2. The unsupplemented chick mash diet, experiments 1 and 3, confirms the previous findings that the progenies of high-protein-casein-fed hens showed a significantly greater mean weight gain than did those of low-protein-corn-fed hens. In experiment 3 the female progenies of the high-protein-casein diet-fed hens outgained the males.

In experiment 2, where the chick diet was unsupplemented and the low-protein-corn maternal diet was supplemented with vitamin B_{12} concentrate, the mean weight gain was significantly greater than the corresponding gain for experiment 1, and these results clearly demonstrate the growth-stimulating effect of transmitted vitamin B_{12}. When the results of experiments 2 and 4 are compared, where vitamin B_{12} concentrate was added to both of the maternal diets the difference between the average means of the two groups of progenies was not statistically significant.

When the chicks' diet was supplemented with B_{12} concentrate, a growth increase was observed only in experiment 1, due chiefly to the female chicks. However, this increase was significantly less than was observed for either sex when vitamin B_{12} was transmitted through the egg to the chick. That is, under the conditions of series I the vitamin B_{12} supplementation was significantly more effective with respect to chick growth when provided in the maternal diet than when provided in the chick diet. When vitamin B_{12} concentrate was added to both the hen and chick feed in experiments 2 and 4, lower mean gains were observed than when it was added only to the diet of the hen.

Dried cow manure as a supplement to the chick diet produced a statistically significant increase in chick growth, in relation to the unsupplemented chick diet, for both sexes in experiment 1 and for the males in experiment 3.

The addition of both vitamin B_{12} and cow manure to the chick mash produced significantly less growth than did cow

TABLE 3

The influence of the maternal diet (consumed for a short period after reversal) on chick growth with a variety of supplements in both maternal and chick diet [1]

EXP. NO.	HEN DIET	HEN DIET USED IN PREVIOUS PERIOD	SEX OF CHICK	MEAN WEIGHT GAIN (NO. OF CHICKS) AND STANDARD ERROR				UNWEIGHTED OVER-ALL MEAN
				Chick mash 1096 without or with supplements				
				None	0.2% B_{12} conc.	5% Dried cow manure	B_{12} and cow manure	
5	Low-protein corn	High-protein casein	♂	121 (11) 11	130 (13) 10	124 (13) 10	144 (15) 10	130 (52) 5
			♀	121 (14) 10	153 (13) 10	115 (11) 11	135 (9) 13	131 (47) 6
			average	121 (25) 8	141 (26) 7	120 (24) 8	140 (24) 8	131 (99) 4
6	Low-protein corn + 0.2% B_{12} conc.	High-protein casein + 0.2% B_{12} conc.	♂	125 (14) 10	148 (14) 10	115 (15) 10	126 (15) 10	128 (58) 5
			♀	108 (15) 10	132 (11) 11	123 (6) 16	127 (12) 11	122 (44) 6
			average	116 (29) 7	141 (25) 8	119 (21) 9	126 (27) 7	125 (102) 4
7	High-protein casein	Low-protein corn	♂	137 (11) 11	166 (17) 9	147 (11) 11	138 (14) 10	147 (53) 5
			♀	136 (18) 9	133 (12) 11	119 (17) 9	133 (14) 10	130 (61) 5
			average	136 (29) 7	150 (29) 7	133 (28) 7	135 (28) 7	139 (114) 4
8	High-protein casein + 0.2% B_{12} conc.	Low-protein corn + 0.2% B_{12} conc.	♂	123 (14) 10	163 (10) 12	98 (6) 16	122 (14) 10	126 (44) 6
			♀	127 (14) 10	133 (19) 9	106 (20) 8	104 (13) 10	118 (66) 5
			average	125 (28) 7	148 (29) 7	102 (26) 9	113 (27) 7	122 (110) 4
Unweighted over-all mean			♂	126 (50) 5	152 (54) 5	121 (45) 6	132 (58) 5	133 (207) 3
			♀	123 (61) 5	138 (55) 5	116 (54) 5	125 (48) 5	125 (218) 3
			average	125 (111) 4	145 (109) 4 [2]	119 (99) 4	129 (106) 4	129 (425) 2 [3]

[1] See footnote 1 in table 2.

[2] See footnote 3 in table 2.

[3] Started with 468 chicks, of which 31 died and 12 lost their identification wing bands.

manure alone in experiments 1, 3 and 4. In experiment 2 only the male chicks' growth was suppressed.

Among the 4 chick diets, the one supplemented with cow manure resulted in a significantly larger over-all mean gain than was observed with the other chick diets. Among the 4 maternal diets, the unsupplemented low-protein-corn produced significantly less over-all mean gain in chick growth than did any of the other maternal diets.

Series II. After reversal of the maternal diets

The results of changing the hens' diets from a low-protein-corn, which they consumed for an extended period, to a high-protein-casein, and vice versa, are summarized in table 3.

After reversal of the maternal diets, the progenies of the high-protein-casein-fed hens (experiment 7) on unsupplemented chick mash outgained the progenies of the low-protein-corn-fed hens (experiment 5) and vitamin B_{12} supplementation to these maternal diets failed to improve chick growth.

However, adding vitamin B_{12} to the chick mash increased appreciably the growth of the progenies of the low-protein-corn-fed hens, but increased only the growth of male progenies of the high-protein-casein-fed hens.

Addition of dried cow manure to the chick mash after reversal of the maternal diets produced no significant improvement in chick growth. Chicks originating from hens fed the high-protein-casein diet + vitamin B_{12} concentrate (experiment 8) gained even less weight with the cow manure supplement in their diet than chicks receiving the unsupplemented mash. Before reversal of the maternal diet the progenies showed the highest weight gain after cow manure supplementation of their feed, while in experiment 8 after reversal a pronounced growth inhibition was noted. Chick growth was not affected by adding both cow manure and vitamin B_{12} concentrate to the chick diet, except for a moderate growth increase shown in experiment 5.

After the reversal of the maternal diet the growth of the progenies during our experimental period did not return to the levels observed before reversal. In both series the eggs used were laid by the same groups of hens (though some weeks later), and the dietary regimen of the chicks in their respective grouping was also the same. The vitamin B_{12} content of the hens' diet in experiments 2, 4, 6 and 8 was practically identical. Assuming that the vitamin B_{12} content of the eggs laid by these hens also remained the same, then the only variable that could account for the differences in growth of the progenies obtained by reversing the maternal diets from corn (experiment 2) to casein (experiment 8) and from casein (experiment 4) to corn (experiment 6) was a change in the transmitted nutrients in the egg. Changes in the egg white protein percentages (Csonka and Jones, '52) and in the cystine and methionine content of the hens' eggs (Csonka et al., '47) brought about by changes in the dietary protein in the feed rations have been shown.

COMMENTS

When cow manure was added as a supplement to the chick diet, the progenies of the unsupplemented high-protein-casein-fed hens reached the highest average weight gain, even higher than that reached by the progenies of the hens fed corn and vitamin B_{12} concentrate (table 2). Analysis showed that the low-protein-corn diet fed to the hens contained 0.1 µg vitamin B_{12} and the high-protein-casein diet 0.56 µg vitamin B_{12} per 100 gm of feed. The average vitamin B_{12} content of the eggs laid by these hens was 0.03 µg and 0.27 µg, respectively. When vitamin B_{12} concentrate was added to the corn- or casein-containing maternal diets, the average vitamin B_{12} content of the eggs increased to 1.8 µg and 1.6 µg, respectively. In view of the lower initial B_{12} content of the egg (0.27 µg for experiment 3 and 1.8 µg for experiment 2), and of the constant vitamin B_{12} content of the chick diet, the differences in chick growth observed do not seem to be related entirely to the vitamin B_{12} content of the diet.

In all experiments when vitamin B_{12} concentrate was added to the chick mash there was a considerably better mean gain with the high-protein-casein maternal diet supplemented or unsupplemented than with the corresponding low-protein-corn diet, although the differences were not always statistically significant.

The foregoing results indicate the existence in the high-protein-casein diet of a growth factor other than vitamin B_{12} which may be related to the dietary protein itself.

A sudden reversal of the maternal diet produced marked changes in the progeny development. The over-all average gain in chick growth for the 16 dietary regimens investigated was 129 gm, a gain significantly less than the 169 gm gain observed before reversal. Moreover, there was a general tendency for lower chick growth when the maternal diets were supplemented with vitamin B_{12} than when they were unsupplemented, in contrast to most of the corresponding results observed before reversal. There was a marked difference in the two series in the relative effect on chick growth of either vitamin B_{12} or cow manure addition to the chick diet. Before reversal the growth response on cow manure supplementation was significantly greater than that with vitamin B_{12} concentrate; after reversal the response to vitamin B_{12} was significantly greater.

It is recognized that the feeding of vitamin B_{12} concentrate may supply other factors in addition to vitamin B_{12}, but whatever is the causative factor, changes in chick growth must be traced back to the egg in view of the fact that the feed composition of the chick's diet in its respective group was held constant.

In a study on the effect of the dietary protein in the maternal diet on the growth of progeny, chickens were chosen as the experimental subject in our work as in the past, and the findings presented here are only presumed to be applicable to mammals, including man.

SUMMARY

1. A high-protein-casein maternal diet resulted in faster growth of progenies than a low-protein-corn diet. From experiments with vitamin B_{12} concentrate in the maternal or chick diet, or with dried cow manure-supplemented chick diets, evidence was obtained to suggest that part of the growth response is due to a transmitted growth factor which differs from vitamin B_{12} and which may be related to the dietary protein.

2. Supplementation of the maternal diet with vitamin B_{12} concentrate for an extended period produced a greater response in the progenies than when the supplement was offered in the chicks' diet.

3. Vitamin B_{12} or dried cow manure supplementation of the chick diet may each act in a growth-promoting or growth-inhibiting fashion on the chick, depending on the maternal diet and on the length of time the hen is on this dietary regimen.

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THE INFLUENCE OF PROTEIN AND ENERGY INTAKES UPON NITROGEN RETENTION IN THE PREGNANT RAT [1, 2]

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FOUR FIGURES

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The primary criterion for evaluating the efficiency of a diet for successful reproduction in the rat, as suggested by Sica and Cerecedo ('48), is the birth weight of the young. However, since the response of the maternal organism to the stress imposed by pregnancy determines, and is inextricably linked with, the condition of the young at birth, it would appear that evaluation of a diet for successful reproduction should have as its first criterion the maternal response, and that fetal size or viability, or both, should be considered as the secondary, albeit extremely important, criterion. In studies such as those conducted by Vinson and Cerecedo ('44), by Nelson and Evans ('47), and by Goettsch ('49), the success of a diet for reproduction was judged by the ability of the female rat to produce successive litters of viable young. These were long-term studies in which the maternal response did receive first consideration.

In our laboratory we have found that the reproductive performance of rats receiving either a 16% or a 25% protein diet was similar when judged on the basis of average maternal

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weight gain during the pregnancy period, or by the size of the litter produced. Although there were slight differences in the average fetal weights attained on each of the diets, those averages were above the minima suggested by Sica and Cerecedo ('48) for use in evaluating the success of diets for reproduction.

Since pregnancy is normally a period of nitrogen acquisition, and since nitrogen acquisition is closely interrelated with the quality and quantity of dietary protein, as well as with the energy level of the diet and with the adequacy of the other essential nutrients, it would appear that nitrogen retention during pregnancy could serve as a suitable criterion of maternal response. This paper is concerned specifically with the levels of nitrogen retention during pregnancy on each of two levels of dietary protein and two levels of calorie intake.

EXPERIMENTAL METHOD

Female rats of the Sprague-Dawley strain were maintained on a diet of commercial laboratory chow [3] until they attained a weight of approximately 200 gm. Estrous cycles were followed by daily vaginal smears and the animals were mated at the appropriate time in the cycle. Mating was confirmed by the presence of sperm in the smear.

On the morning mating was confirmed, the animals were placed in individual cages and offered one of the three experimental diets shown in table 1. The diet designated as "R" was the commercial chow, which was finely ground in order to permit careful weighing and to reduce spillage. Diets R-1 and R-2 were modifications of the ground chow in which the percentage of protein was reduced by dilution with fat [4] and carbohydrate [5] in order to obtain two diets of like protein content but with a high and low calorie value. Twenty grams of fat and 14 gm of carbohydrate were added to each 66 gm of chow for the high calorie diet (R-1), and 2 gm of fat and

[3] Purina.
[4] Crisco.
[5] Cornstarch.

32 gm of carbohydrate were added for the low calorie diet (R-2).[6] The resulting percentage composition of the diets and the calorie and non-protein calorie values per gram are given in table 1. The diets were fed ad libitum and records were kept of the quantity consumed by each animal during each week of the pregnancy period. Nitrogen analyses of the diets

TABLE 1

Composition of diets[1]

DIET	PROTEIN	FAT	CARBO-HYDRATE	CAL./GM	NON-PROTEIN CAL./GM
	% [2]	%	%		
R[3]	25	6	47	3.4	2.4
R-1	17	24	45	4.6	4.0
R-2	16	6	63	3.7	3.1

[1] Percentages of fiber and moisture are not included.

[2] By analysis.

[3] Except for the protein value, the composition of diet R was obtained from the manufacturer's analysis.

were made for computation of nitrogen intake. Calorie intakes were calculated from food intakes, using the conventional equivalents.

Pans containing boric acid-infiltrated filter paper were placed beneath each cage for urine collection. Feces were removed from the paper each day and held in 50% sulfuric acid until the week's collection for each animal was completed. At the end of each of the three weeks, urine samples were prepared by repeated washings of the pan and filter paper with dilute sulfuric acid and the washings were made up to volume. Fecal samples were made up to weight with water and homogenized in a Waring Blendor. Duplicate

[6] Computations based on the manufacturer's analysis of the chow indicate that the amino acid and vitamin content of the modified chow diets not only met, but in most instances greatly exceeded, the requirements suggested for the rat. All of the mineral constituents of the diet met or exceeded the suggested requirements, with the exception of iron and iodine; 92% of the iron requirement was supplied and 83% of the iodine.

aliquots were analyzed for nitrogen content using the Kjeldahl method with a selenium catalyst.

In order to provide a basis for comparison that would be independent of variations in size and weight, all nitrogen intake and retention figures were calculated as milligrams per kilogram per day. The weight in kilograms of each animal for each weekly period was obtained by averaging the weights recorded for the first and last days of the week. Energy intake was calculated as non-protein calories per square meter per day using the formula, $S.A. = 11.36 \times W^{2/3}$,

TABLE 2

Average maternal weight gain, litter size and fetal weight

DIET GROUP	MATERNAL WEIGHT GAIN		NUMBER IN LITTER	FETAL WEIGHT
	Total	2nd & 3rd wks.		
	gm	*gm*		*gm*
R	138	111	10.0	5.6
R-1	131	102	10.1	5.2
R-2	130	108	10.1	6.0

as suggested by Carman and Mitchell ('26). The decision to estimate energy intake on the non-protein calorie content of the diet was based on the assumption that dietary protein used as a source of amino acids for growth cannot simultaneously serve as a source of energy in the diet.

The values obtained for the first week of pregnancy are not included in the data presented, since this period was considered as an adjustment period for the two groups of animals receiving the lowered protein intake.

The data on comparative birth weights and birth lengths of the young will be presented in a subsequent paper.

RESULTS

The similarity in the average maternal weight gains during pregnancy and in the average litter size and fetal weight for animals in each of the three diet groups is shown in table 2.

The mean daily nitrogen intakes, non-protein calorie intakes and nitrogen retentions for the three diet groups during the second week of pregnancy are presented in table 3. The mean daily nitrogen intake on the high protein diet (R) was approximately twice that of either of the diets containing the lower protein level. However, the mean daily nitrogen retentions bore no obvious relationship to the levels of nitrogen intake, other than the fact that the highest intake led to the highest absolute nitrogen retention. No relationship

TABLE 3

Mean daily nitrogen intakes, non-protein calorie intakes and nitrogen retentions during pregnancy

DIET GROUP	NO. OF RATS	NITROGEN INTAKE		NON-PROTEIN CALORIE INTAKE N.P.Cal./m²		NITROGEN RETENTION		
		mg/kg	σm [1]		σm	*mg/kg*	σm	%
				Second week				
R	25	3,204	± 112	1,062	± 22	431	± 33	13.5
R-1	10	1,747	± 47	1,439	± 40	377	± 46	21.6
R-2	10	1,612	± 20	1,138	± 14	199	± 29	12.3
				Third week				
R	25	2,945	± 69	1,042	± 22	619	± 33	21.0
R-1	10	1,632	± 53	1,424	± 45	456	± 27	27.9
R-2	10	1,581	± 41	1,184	± 27	423	± 36	26.8

[1] Standard error of the mean.

whatsoever could be observed between the percentages of nitrogen intakes retained and the levels of protein in the diets. However, it should be observed that the highest non-protein calorie intake was associated with the highest percentage of nitrogen retention.

The nitrogen intakes for the second week of pregnancy are plotted against nitrogen retentions in figure 1. It will be noted that the correlation coefficients are statistically significant only for diet R-1 and that the lowest degree of correlation occurs for the high protein group (R). The results are similar when non-protein calorie intakes are plotted against nitrogen re-

tentions (fig. 2), the highest degree of correlation occurring on diet R-1 and the lowest on diet R. The degree of correlation parallels the level of non-protein calorie intake for the three groups, the highest correlation occurring for the group with the highest non-protein calorie intake.

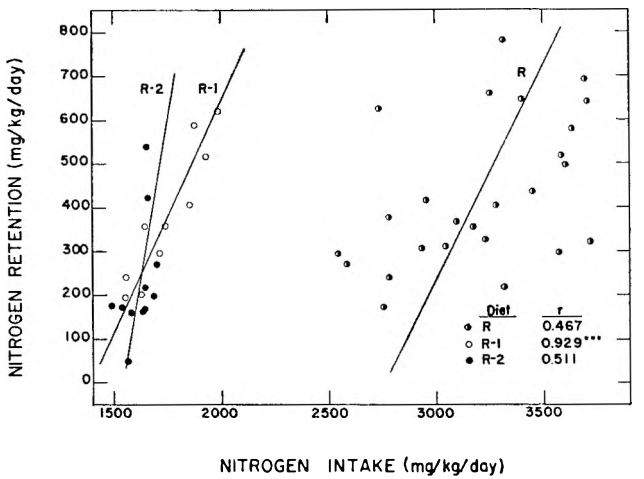

Fig. 1 Regression lines and correlation coefficients for nitrogen intakes and nitrogen retentions during the second week of pregnancy.

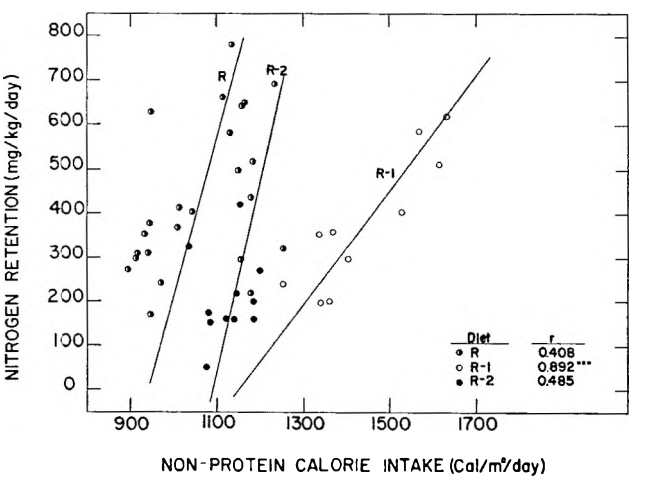

Fig. 2 Regression lines and correlation coefficients for non-protein calorie intakes and nitrogen retentions during the second week of pregnancy.

Table 3 also presents the mean daily nitrogen intakes, non-protein calorie intakes and nitrogen retentions for the three diet groups during the third week of pregnancy. The nitrogen intake figures calculated on the basis of milligrams per kilogram per day show a decrease when compared to the intakes for the second week. This occurred because the magnitude of the increase in body weight exceeded that of the increase in grams of ration consumed by each group. In other words, although total food consumption, and therefore nitrogen intake, increased during the third week, they decreased when calculated per unit of body weight. The increase in actual food consumption was greatest for the animals on diet R-2; this becomes evident from the smaller decrease in the mean milligrams of nitrogen consumed per kilogram of body weight when the second and third weeks are compared. This increased rate of food consumption on diet R-2 is reflected to an even greater extent in the calculation of non-protein calorie intake per unit of surface area, since the mean figure for this group shows an increase for the third week while the means for the other two groups show a decrease.

During this final week of pregnancy the percentages of the nitrogen intake retained and the actual quantities retained showed an increase over the second week for all groups. However, a difference in the rate of increase among the groups may be observed. In the third week, as in the second, the highest level of nitrogen retention was associated with the highest level of nitrogen intake, whereas the highest percentage of nitrogen intake retained was associated with the highest level of non-protein calories consumed.

When nitrogen intakes or non-protein calorie intakes for the last week of pregnancy are plotted against nitrogen retentions (figs. 3 and 4), the highest degrees of correlation are again observed for diet R-1. However, it should be noted that, whereas the degree of correlation during the third week was less than during the second week for the high protein group (R) in both instances, it had increased for the low protein-low calorie group (R-2). Again, the degree of cor-

relation paralleled the level of non-protein calorie intake, and the stronger correlations observed during the third week of pregnancy for diet R-2 were associated with the increase in both nitrogen and non-protein calorie consumption per body unit by that group in the final week of pregnancy.

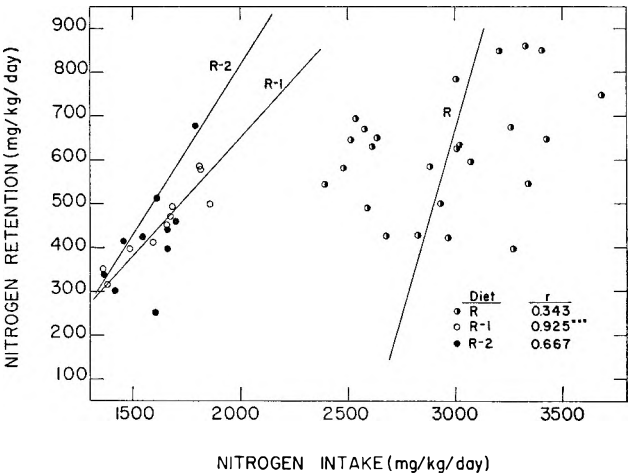

Fig. 3 Regression lines and correlation coefficients for nitrogen intakes and nitrogen retentions during the third week of pregnancy.

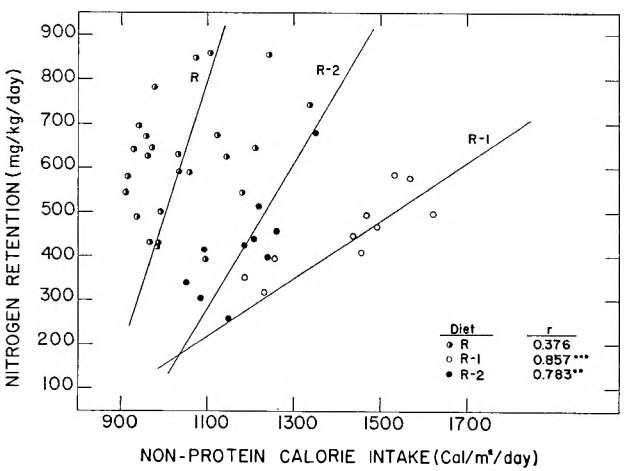

Fig. 4 Regression lines and correlation coefficients for non-protein calorie intakes and nitrogen retentions during the third week of pregnancy.

DISCUSSION

The second week of gestation in the rat is the period of organogenesis, whereas the third week is the period of rapid fetal growth. Unless the diet is deficient, it is during the third week that retention of nitrogen reaches the highest level attained during pregnancy.[7] In addition to supplying the needs of the fast-growing young, the retained nitrogen is also required for the growth of maternal tissue and probably for a reserve to help meet the needs of lactation. Since the stress imposed by growth is not as great during the second week as during the third week of pregnancy, a difference in the metabolic needs of the maternal organism might be expected, as well as a difference in the efficiency with which dietary nutrients are utilized during these two periods.

In order to compare more effectively the performance of the animals on the different protein and calorie levels used in this study, we propose the use of diet R-2, the low protein-low calorie diet, as the baseline for comparison. We can then judge the response of such animals to the addition of protein (diet R), or to the addition of non-protein calories (diet R-1). Further, by comparing the low calorie-high protein diet (R) with the high calorie-low protein diet (R-1), the relative limitations imposed by protein and calories may be judged.

Table 4 presents a comparison of the mean daily values for nitrogen intakes, non-protein calorie intakes and nitrogen retentions among the diet groups for each of the last two weeks of pregnancy. Using the low calorie-low protein diet as the suggested baseline, it may be observed that the addition of protein leads to a very highly significant increase in nitrogen retention. This is an expected finding and has been shown to occur in growing rats (Kao, Conner and Sherman, '41; Bosshardt et al., '48), and in adult rats during rehabilitation (Benditt et al., '48b). When the protein level is kept at the baseline and non-protein calories are added (R-1), there is again a highly significant effect on nitrogen retention. It

[7] Unpublished data from this laboratory.

has been shown by Benditt et al. ('48a) that non-protein calories must reach a critical level of 1,240/m²/day in the protein-depleted rat in order to supply the maintenance needs of the animal along with sufficient excess to allow for maximum utilization of the ingested protein for tissue synthesis. At caloric intakes below this critical level, the animal must resort to burning protein for energy purposes. Bosshardt

TABLE 4

Comparison of mean daily values for nitrogen intakes, non-protein calorie intakes and nitrogen retentions during pregnancy

	LOW CALORIE		LOW PROTEIN		LOW CAL. HIGH PRO.	HIGH CAL. LOW PRO.
	Low pro. R-2	High pro. R	Low cal. R-2	High cal. R-1	R	R-1
			Second week			
Nitrogen intake [1]	1,612	3,204 [4]	1,612	1,747	3,204 [4]	1,747
Non-protein calorie intake [2]	1,138 [3]	1,061	1,138	1,439 [4]	1,061	1,439 [4]
Nitrogen retention [1]	199	431 [4]	199	377 [3]	431	377
			Third week			
Nitrogen intake	1,581	2,945 [4]	1,581	1,632	2,945 [4]	1,632
Non-protein calorie intake	1,184 [4]	1,042	1,184	1,424 [4]	1,042	1,424 [4]
Nitrogen retention	423	619 [4]	423	456	619 [4]	456

[1] Milligrams per kilogram.

[2] Calories per square meter.

[3] P greater than 0.01 according to Student's "t" test.

[4] P greater than 0.001 according to Student's "t" test.

et al. ('46) suggest a similar figure, 1,250 Cal./m²/day, as the critical level for the growing rat. Examination of the mean non-protein calorie intakes for diets R and R-1, both of which resulted in a significantly higher nitrogen retention than the baseline diet, show that the non-protein calorie intake provided by diet R was below the suggested critical level required for protein synthesis, whereas ample non-protein calories were provided by diet R-1. Further, since there is no statistical significance to the difference in nitrogen reten-

tion obtained on these two diets, it would appear that a portion of the protein supplied by diet R was being diverted to meet energy needs. It is suggested, therefore, that the factor limiting nitrogen retention in the baseline diet (R-2) during the second week of pregnancy was the supply of non-protein calories, rather than the level of protein.

During the third week of pregnancy, the period of rapid growth, the data provide a picture that is somewhat different. Again using the low protein-low calorie diet (R-2) as the baseline for comparative purposes, table 4 reveals that an increased level of protein in the diet leads to a very highly significant increase in the level of nitrogen retention. This finding is similar to the performance during the previous week of gestation. However, the increase in the efficiency with which the animals on the baseline diet were able to utilize the protein provided (an increase from 12.3% of the nitrogen intake retained to 26.8%, as shown in table 3), appears to be explained by the aforementioned increase in non-protein calories consumed per unit of surface area by this group during the third week. This non-protein calorie level approaches more closely the suggested critical level and appears to have spared some of the dietary protein from being used for energy.

When protein is kept at the baseline level and non-protein calories are added to the diet, as in diet R-1, the difference in the level of nitrogen retention is very slight and of no significance statistically. This is in contrast to what occurred during the second week. It appears that during this period of rapid growth calories are no longer the factor limiting nitrogen retention; rather, it is the level of nitrogen in the diet that is imposing the limitation at this time. This postulate is strengthened by a comparison of diets R and R-1. During the second week, when non-protein calories were limiting, there was little difference in the nitrogen retention on these two diets. However, during the third week, when protein appears to have been the factor limiting nitrogen retention, there was a difference of high statistical signifi-

cance in the levels of nitrogen retention attained, when diets R and R-1 are compared. It would appear, therefore, that the beneficial effect of an increase in the non-protein calorie intake was prevented by the inadequacy of the protein intake during the third week. The data indicate that the nitrogen intake during the third week of pregnancy should be somewhat higher than the 1,632 mg/kg/day provided by diet R-1, although not necessarily as high as the 2,945 mg/kg/day in diet R, if the efficient use of dietary protein is insured by an adequate quantity of non-protein calories. Further, the results indicate that for the conditions of this study, nitrogen retention is a more sensitive criterion for evaluating a diet for successful reproduction than is the criterion of fetal weight, since significant differences were observed in nitrogen retention whereas fetal weights were similar.

SUMMARY

A comparison was made of nitrogen retentions in groups of laboratory rats on two levels of dietary protein and two levels of energy intake during the second and third weeks of pregnancy.

Differences in the metabolic needs of the maternal organism between the second and third weeks of pregnancy were reflected in the utilization of the protein and energy supplied by the diets.

On the low protein-low calorie diet, the factor limiting nitrogen retention during the second week of pregnancy appeared to be the non-protein calorie intake, whereas during the third week the protein intake appeared to be limiting.

On the high protein-low calorie intake, there was little correlation of either nitrogen intake or non-protein calorie intake with nitrogen retention during either week of pregnancy, and the data indicate the use of dietary protein for energy purposes.

During each of the weeks of pregnancy studied, the highest percentage of nitrogen retained was associated with the highest non-protein calorie intake. Strong correlations of nitrogen

intake or non-protein calorie intake with nitrogen retention were apparent when the non-protein calorie intake was high.

It is suggested that nitrogen retention during pregnancy, rather than the birth weight of the young, be considered the criterion of the adequacy of a diet for reproduction in the rat.

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AMINO ACID DEFICIENCIES OF CASEIN AS A SOURCE OF PROTEIN FOR THE CHICK [1]

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Students of poultry nutrition are using synthetic diets extensively and future progress depends largely on having a basal diet that is well adapted for its purpose. Casein is the protein most frequently used in synthetic diets, and its adequacy has been a source of concern over a period of several years. The publications in this field are too numerous for a detailed review and only a few will be mentioned.

Arnold, Kline, Elvehjem and Hart ('36) demonstrated that as a source of protein for chicks casein is grossly deficient in arginine. According to Almquist, Mecchi and Kratzer ('41), there is under some circumstances a definite increase in the rate of gain by chicks when 1% or more of glycine is included in the diet. If 1% of arginine was added to the diet, the chicks grew still more rapidly, and the most rapid gains were obtained when arginine and glycine were added simultaneously. When 1.5% of creatine was added to the diet, the rate of gain was practically the same as on addition of the combination of arginine and glycine. Another important contribution was made by Briggs, Mills, Elvehjem and Hart ('42). Under their experimental conditions the rate of gain

[1] Taken from a thesis presented by Mr. Wietlake in partial fulfillment of the requirements for the degree of Master of Science. Supported in part by a grant from Merck and Co., Rahway, New Jersey. Contribution from the Missouri Agricultural Experiment Station. Journal Series 1379.

was practically doubled when 0.3% of cystine was added with a combination of arginine and glycine.

It is impractical because of the cost to use arginine or creatine in large-scale nutrition studies, and it is the practice to include 10% of gelatin in casein diets, as described by Almquist, Mecchi and Kratzer ('41), in an attempt to provide adequate amounts of arginine and glycine. Casein also contains an insufficient quantity of the sulphur-containing amino acids and this deficiency is most commonly remedied by including methionine in the diet. Apparently the adequacy of casein when supplemented in this manner has never been questioned, and it is of considerable importance to determine whether or not the biological value of the casein-gelatin-methionine type of diet is as high as previously supposed. Information on this point should make it possible to supply the combination of amino acids that is most effective for at least two important problems: (1) rapid growth of the chick, and (2) a diet suitable for investigations of unrecognized nutrients. A preliminary report on our observations was published recently (Hogan et al., '53).

EXPERIMENTAL AND RESULTS

The chicks employed were White Leghorns and within one day after hatching they were wing-banded and divided among the experimental groups. So far as was possible, chicks of the same weight were distributed evenly in the various groups. As a rule the rations were tested several times with a small number of chicks, rather than once with a larger number. As the investigation proceeded some of the findings were unexpected and the original plan was modified. As a result, some of the trials were repeated more often than others. At the end of a 4-week experimental period the chicks were graded as to feathering, on a scale from one to 5. One is the lowest grade, and 5 represents perfect feathering. The averages of the groups were converted into percentages. The percentage of abnormal gait, a type of leg weakness, was also calculated for each group. The affected chicks do not

stand up for any length of time and when they try to walk they lack coordination and have a stilted, hurried gait, as if the muscles are so weak that one leg is unable to support the body weight. Presumably this is the abnormality described by Almquist, Mecchi and Kratzer ('41) as "a condition of

TABLE 1

Composition of basal ration, No. 3237

INGREDIENTS	AMOUNT	VITAMINS ADDED PER 100 GM [4]	AMOUNT
	gm		*mg*
Casein [1]	35.0	Thiamine HCl	1
		Riboflavin	1
Cerelose	46.5	Pyridoxine HCl	1
		Ca-pantothenate	3
Cellulose	3.0	Niacin	5
		Choline Cl	200
Soybean oil	10.0	Inositol	50
		Folic acid	0.2
Salts [2]	5.0	Vitamin E	2.5
		Vitamin K	2.5
DL-Methionine [3]	0.5	Biotin	20 μg
		Vitamin B_{12}	3 μg
		Vitamin A	2,000 I.U.
		Vitamin D	433 I.U.

[1] Prepared by the method, with slight modifications, of McCollum et al. ('22).

[2] Described by Richardson and Hogan ('46).

[3] Courtesy of the Dow Chemical Co., Midland, Mich.

[4] A concentrate of vitamins A and D was purchased from Distillation Products Industries, Rochester, N. Y. Folic acid was supplied by the Lederle Laboratories, Pearl River, N. Y. All other vitamins were supplied by Merck and Co., Rahway, N. J.

general weakness and muscular attenuation," and by Hegsted, Hier, Elvehjem and Hart ('41b) as a "high stepping, stilted gait."

The chicks described in each of the following tables were from the same hatch and were under observation at the same time. This will explain why, when a ration appears in more than one table, the number of chicks that received it may vary. The chicks were housed in an electrically heated battery

on raised screen floors and were allowed food and water ad libitum. The basal diet is described in table 1. When gelatin was included in the basal diet, it replaced an equal weight of casein. When amino acids or creatine were included, they replaced an equal weight of cerelose. In order to conserve space, only the variable constituents, which do not appear in table 1, are included in subsequent tables.

TABLE 2

Effect on the growth rate of adding arginine, glycine or creatinine to the basal diet

		RATION			
		3237	3232	3238	3233
Glycine [1]	%	0	1.5	0.0	0
L-Arginine HCl [2]	%	0	0	0.8 (0.66)	0
Creatine [3]	%	0	0	0.0	1.5
Abnormal gait	%	14	32	0	10
Feathering	%	21	30	65	54
Ave. weight at 4 weeks					
Males	gm	156 (10)[4]	175 (7)	296 (12)	305 (11)
Range	gm	104–224	94–260	248–372	146–424
Females	gm	113 (19)	185 (21)	316 (15)	249 (20)
Range	gm	78–198	74–348	222–416	96–370

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

[2] Courtesy of Merck and Co., Rahway, New Jersey. The amino acid was supplied as the monohydrochloride. The corresponding amounts calculated as the free base are shown in parentheses in table 2, 3, 4 and 5.

[3] Distillation Products Industries, Rochester, N. Y.

[4] The figures in parentheses following the average weights show the number of chicks observed.

The first feeding trials to be described were merely to confirm the reports of other investigators. The basal diet, No. 3237, contained no supplement. Each of the other three diets described in table 1 was supplemented with a different member of the group — arginine, glycine or creatine. The results are shown in table 2.

As one would expect, the rate of growth on the basal diet, 3237, was exceedingly poor, and in addition 14% of the chicks

had an abnormal gait. The feathers of the lighter chicks were given a low score and were rough and broken. When glycine was included in the diet, the improvement was not marked. There was a small increase in the mean weight, but the feathering was not greatly improved and the incidence of "quickstep" doubled. This agrees with the observations of Almquist et al. ('40), and of Hegsted, Hier, Elvehjem and Hart ('41b), that at times the addition of glycine alone to the diet brings about no important improvement when casein is the source of protein. Probably the most noteworthy feature of the results with ration 3232 was the extreme variability. Five females weighed less than 100 gm, and three weighed over 300 gm. It is commonly supposed that a deficiency of an essential amino acid retards the synthesis of new tissues under all circumstances and makes it impossible for any chick to attain a normal weight. The three high weights suggest that arginine is essential for the synthesis of some unique biochemical substance, apart from the construction of tissue proteins. One could suppose that the variability in response to a deficiency of this unique substance is as great as when a vitamin is in short supply.

Arginine alone was added at two different levels, 0.5 and 0.66%. The difference between the average weights was slight, and not statistically significant,[2] hence the results are combined and ascribed to ration 3238. It will be noted that the inclusion of arginine brought about a striking improvement, and the weights obtained were comparable to those published in the literature, when chicks presumably consumed an adequate diet of natural foodstuffs. The feathering was vastly improved and abnormal gait was eliminated. It is evident that, as has been stated previously, casein is grossly deficient in arginine. Ration 3233 contains 1.5% of added creatine and this addition brought about marked improvement in weight, in feathering, and in the incidence of abnormal gait. The weights on rations 3238 and 3233 were of the same order of

[2] The assistance of Dr. Laura M. Flynn and Dr. M. S. Zuber with the statistical analysis is gratefully acknowledged.

magnitude but the higher weights of the females on ration 3238 suggest that 0.66% of arginine improved the basal diet more than did 1.5% of creatine.

Since maximum growth rates were not obtained when the supplements were added one at a time, as in table 2, they were added in combinations of two in the next series. The results are shown in table 3.

TABLE 3

Effect on the growth rate of adding combinations of arginine, glycine and creatine to the basal diet

		RATION [1]			
		3235	3234	3236	3251
Gelatin	%	0	0	0	10
L-Arginine HCl	%	0.6 (0.5)	0	0.6 (0.5)	0
Glycine	%	1.5	1.5	0	0
Creatine	%	0	1.5	1.5	0
Abnormal gait	%	4	12	0	0
Feathering	%	65	56	71	61
Ave. weight at 4 weeks					
Males	gm	285 (13)	310 (12)	339 (15)	332 (13)
Range	gm	174–444	154–452	166–480	140–444
Females	gm	299 (12)	263 (14)	310 (11)	318 (13)
Range	gm	164–366	74–388	248–360	194–412
Comparative final weight		90	88	100	100
Standard deviation		± 27.5	± 33.0	± 21.7	± 23.2

[1] The "t" value for significance of difference at 5%:

Rations	Found	Required
3236 > 3234	1.60	2.01.

There was a high degree of variability within lots, and this makes it impossible to give the rations definite comparative ratings. It seems certain that ration 3235, which contained 0.5% of arginine and 1.5% of glycine, was not superior to ration 3238 in table 2, which contained arginine alone with no glycine. In fact, the chicks which consumed ration 3238 were the heavier, though the difference was small. Ration 3234 contained a combination of glycine and creatine, and

ration 3236 contained a combination of arginine and creatine. The data indicate that of these two rations, 3236 is the better, but the difference had little or no statistical significance. Ration 3251 contained 25% of casein and 10% of gelatin, and apparently it supported about the same rate of growth as did ration 3236. However, none of the rations was entirely adequate, as is shown by the extreme ranges in weight and

TABLE 4

Effectiveness of gelatin as a supplement to the basal diet, in comparison with a combination of arginine, glycine and creatine

		RATION [1]	
		3252	3251
Gelatin	%	0	10
L-Arginine HCl	%	0.6 (0.5)	0
Glycine	%	1.5	0
Creatine	%	1.5	0
Abnormal gait	%	0	9
Feathering	%	84	62
Ave. weight at 4 weeks			
Males	gm	392 (23)	319 (19)
Range	gm	202–504	118–444
Females	gm	337 (28)	284 (24)
Range	gm	206–426	138–412
Comparative final wt.		121	100
Standard deviation		± 19.0	± 28.8

[1] The "t" value for significance of difference at 1%:

Rations	Found	Required
3252 > 3251	4.13	1.99.

by the large standard deviations. Under these circumstances it did not seem highly important to determine their relative order of merit.

Since the sex of the chicks was not determined at the beginning of the feeding trials, there were usually unequal numbers of males and females on the rations to be compared. This disparity could interfere with the statistical treatment, and in an attempt to avoid that complication the weights of the chicks were converted to "comparative final weights."

They are expressed as percentages of the mean weight of the chicks on one of the rations selected as a base. In table 3 the base ration was 3251, and the mean weight of the males was 332. As an example, the weight of each male in ration 3235 was calculated as a percentage of 332. In like manner, the weight of each female was calculated as a percentage of 318. The mean of the combined percentages was 90, and the standard deviation of the combined percentages was $\pm$ 27.5. Data calculated in this manner appear in tables 3, 4 and 5.

Since the basal diet was not made adequate by a combination of any two of the three supplements, arginine, glycine and creatine, it was decided then to make up a new ration, No. 3252, which contained all three of them simultaneously. The resulting data are shown in table 4.

Ration 3252 was much superior to ration 3251. The weights at 4 weeks of age were surprisingly high, the feathering was excellent, and there were no cases of abnormal gait. The standard deviation is undesirably high, but one cannot be certain that this is due to an inadequate diet. It seems clear that the casein-gelatin-methionine type of diet is deficient in one or more important amino acids, and that the deficiency was remedied by a combination of arginine, glycine and creatine. Our data are in conformity with earlier reports that the basal diet is improved by arginine alone or by creatine alone, but the degree of improvement when glycine was added to the diet was not marked. It will be observed that in three out of 4 possible comparisons, if tables 2, 3 and 4 are combined, there was some increase in the rate of gain when glycine was included in the ration, but the only difference of any consequence was between rations 3236 and 3252.

From a nutritional standpoint, arginine and creatine are in large measure interchangeable. The next objective was to determine how much creatine, if any, is required for optimum growth, and whether or not it can be entirely replaced by arginine. The data are shown in table 5.

Ration 3235 contained 0.5% of arginine and no creatine. When 1.5% of creatine was added to this diet, as in ration

TABLE 5

The effectiveness of creatine as a supplement to the basal diet depends on the supply of arginine

		RATION [1]					
		3235	3309	3308	3252	3310	3389 [2]
L-Arginine HCl	%	0.6 (0.5)	0.6 (0.5)	0.6 (0.5)	0.6 (0.5)	1.5 (1.24)	1.5 (1.24)
Glycine	%	1.5	1.5	1.5	1.5	1.5	1.5
Creatine	%	0.0	0.5	1.0	1.5	0.0	1.5
Abnormal gait	%	10	0	0	0	0	0
Feathering	%	68	59	69	79	78	73
Ave. weight at 4 weeks							
Males	gm	353 (11)	339 (6)	317 (8)	423 (9)	410 (7)	381 (6)
Range	gm	176–438	170–436	148–534	344–504	358–442	336–408
Females	gm	357 (6)	333 (10)	347 (8)	336 (8)	341 (9)	369 (3)
Range	gm	254–444	160–446	292–480	250–426	222–408	354–384
Comparative final weight		91	92	89	100	100	97
Standard deviation		± 21.7	± 28.8	± 27.9	± 13.8	± 13.1	± 11.2

[1] The "t" value for significance of differences at 5%:
Only the males were used in these calculations

Rations	Found	Required
3252 > 3235	2.23	2.10
3252 > 3308	2.31	2.13
3252 > 3309	1.99	2.16

[2] One trial only. All other rations were compared in two trials.

3252, there was a marked improvement in the rate of growth. Rations 3308 and 3309 apparently contained less than the optimum amounts of creatine, unless the ration was changed in other respects. It will be noted that the females on rations 3235, 3308 and 3309 were as heavy as those on ration 3252. The differences between the weights of the males are either significant or they closely approach significance, at the 5% level. One should have more evidence, though, before concluding that there is a sex differential in the response to a deficiency of arginine.

Ration 3310 contained no creatine but the amount of arginine was increased from 0.5 to 1.24%, and the chicks grew as rapidly as they did on ration 3252. The results with ration 3389 supply additional evidence on this point. This diet contained 1.5% of creatine in addition to 1.24% of arginine, but the chicks did not grow more rapidly than they did on ration 3310, which contained no creatine. One would conclude that creatine can replace most but probably not all of the arginine required. If the diet contains the optimum amount of arginine, there is no further improvement when creatine is added.

DISCUSSION

The large amount of arginine that was necessary to correct the deficiency in casein was an unexpected observation. The required amount was over 0.66% and probably less than 1.24%. The casein we used contained 13.8% of nitrogen and according to Block and Bolling ('51) it would therefore contain approximately 1.3% of arginine. According to Almquist and Merritt ('50), the diets we used should contain 1.8% of arginine in order to meet the requirement of the chick. At that rate, 0.66% of additional arginine, as in diet 3238, should be enough, but in our experience a somewhat larger amount is required. Presumably our higher estimate is due to different experimental conditions. Our chicks were White Leghorns, which feather rapidly, and according to Hegsted, Briggs, Elvehjem and Hart ('41a) they have a higher requirement for arginine than do the heavier breeds which

feather slowly, such as were used by Almquist and his associates. Moreover, we began with chicks that had just hatched and the experimental period covered the interval of most rapid growth, both of feathers and of total body. Almquist and his co-workers began with chicks that were 4 weeks old, after the feathers were well developed. Under our experimental procedure a higher arginine requirement is to be expected.

TABLE 6

Distribution of chicks in various weight groups

RATION NO. AND SUPPLEMENTS	EQUIVALENT FINAL WEIGHTS			
	37–70	71–110	111–150	151–158
	%	%	%	%
3252 Arginine, glycine, creatine	2.0	17.6	76.4	4.0
3251 Gelatin	18.5	39.5	42.0	0.0

The above explanation would also apply to the failure of 10% of gelatin to maintain a higher nutritional status. According to Block and Bolling ('51), 10% of gelatin would supply 0.82% of arginine, and in our experience that is not enough.

In a comparison of rations 3251 and either 3252, 3310 or 3389, there are 4 differences that are worthy of some comment: (1) the chicks that consumed ration 3251 had lower average weights; (2) the standard deviation and the range of the weights were larger; (3) about 10% of the chicks had an abnormal gait; (4) the feathering was inferior. Presumably the abnormal gait is a symptom of a specific deficiency, and all of the symptoms are interrelated. One would suppose that the exceedingly low weights sometimes observed began with simple failure to consume a normal amount of food, as will happen in time when any deficiency becomes severe enough to lower the nutritional status. The failure was probably intensified, though, by the abnormal gait and the difficulty in reaching the food containers.

It seemed that the high degree of variability on ration 3251 was by itself weighty evidence of dietary inadequacy and table 6 was prepared as an illustration.

The equivalent final weights were divided into 4 groups and the percentages of chicks that fell in these groups were calculated. As is shown in table 6, three-fourths of all the chicks on ration 3252 fell in the percentage range 111 to 150, and almost one-fifth fell in the range 71 to 110. Only 6% fell outside of these ranges. In strong contrast to this record, only 42% of the chicks on ration 3251 fell in the range of 111 to 150. Almost as large a number fell in the range 71 to 110, and 18.5% fell in the lowest range, 37 to 70. In our experience a high degree of variability is, as a rule, a result of dietary inadequacy. Since the weights attained by chicks on rations 3252, 3310 and 3389 were exceptional for White Leghorns, it should be emphasized that these diets did not contain antibiotics or any known source of unrecognized growth factors. It is planned to study the effect on growth when these supplements are added to such a ration as 3252 or 3310.

The biochemical functions of arginine, glycine and creatine are obscure. Apparently creatine can replace part, but not all, of the arginine that is required. The response to glycine was relatively slight.

SUMMARY

A synthetic diet which contained 35% of casein and 0.5% of methionine was supplemented with arginine, glycine and creatine. When these were added singly the improvement was slight with 1.5% of glycine, and large with 0.5% of arginine or with 1.5% of creatine. When the rations were deficient in arginine the feathers were imperfect and some of the chicks had an abnormal gait. Weights of the same order of magnitude were obtained with arginine alone and with combinations of arginine and glycine, glycine and creatine, and arginine and creatine; also with a combination of 25% casein and 10% gelatin. The range in weight and the

standard deviations of the weights indicated that all of the rations previously mentioned were in some degree inadequate.

When casein alone was supplemented with 0.5% arginine, 1.5% glycine and 1.5% creatine simultaneously, the average weight of the chicks was exceptionally high for White Leghorns, the feathers were excellent, and there were no cases of abnormal gait. When the amount of arginine was increased to 1.24% and creatine was omitted, there was no reduction in the average weight.

A combination of 25% casein and 10% gelatin was partially inadequate as a source of protein for the chick.

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EXPERIMENTAL VARIABLES IN PREDICTING PROTEIN MINIMA FOR RATS [1]

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TWO FIGURES

(Received for publication October 9, 1953)

Melnick and Cowgill ('37) described a method of estimating minimum protein requirements for nitrogen equilibrium in dogs which made use of a linear relationship between nitrogen balance and per cent protein calories in the diet. Using a modification of this method, certain variations in patterns of nitrogen metabolism and weight change in rats which have some bearing on the general problem of estimating the protein needs of animals have been observed.

EXPERIMENTAL

Sprague-Dawley albino rats weighing 150 gm were used in this study. The composition of the diets is shown in table 1. Three consecutive 11-day feeding periods consisting of a 4-day adjustment and a 7-day balance period were used throughout the experiment. Weight change, caloric intake, nitrogen intake and nitrogen balance were obtained for individual rats for each 7-day balance period. Data reported are based on individual rat responses expressed as units per gram weight at the beginning of the period.

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[2] Submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy, School of Graduate Studies, Michigan State College.

The basic feeding pattern used throughout the study is shown in table 2. This was a cross-over design similar to that described by Cochran et al. ('41). Each of the three experiments reported here consisted of three groups of 4 rats (a total of 12 rats) which were fed three diets chosen from table 1 in different sequence. Statistical analyses for

TABLE 1

Composition of experimental diets

CONSTITUENTS	DIET				
	A	B	C	D	E
Starch	73	71	69	63	61
Sucrose	10	10	10	10	10
Agar agar	2	2	2	2	2
Wesson salts [1]	4	4	4	4	4
Lard	4	4	4	4	12
Cod liver oil	3	3	3	3	3
Vitamin oil [2]	3	3	3	3	3
Vitamin starch [2]	1	1	1	1	1
Dried defatted whole egg	0	2	4	10	4
Total	100	100	100	100	100
Per cent protein (N × 6.25)	0.56	1.98	3.24	6.86	3.03
Calories/gm (direct calorimetry)	4.10	4.07	4.14	4.30	4.65

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

[2] Furnished 1.25 mg alpha-tocopherol, 0.15 mg me-napthoquinone, 2.8 mg Ca pantothenate, 1.0 mg niacin, 0.5 mg riboflavin, 0.4 mg thiamine, 0.5 mg pyridoxine, 20.0 mg *p*-amino benzoic acid, 20.0 mg inositol, 20.0 mg ascorbic acid, 50 mg choline per 100 gm diet.

evaluating the effects of experimental variables on rat responses to diets were based on this rotation of feeding.

Further details of the experimental and statistical methods employed are described by Kelley ('52).

RESULTS

Significance of urinary nitrogen in nitrogen metabolism

Table 2 shows the mean urinary nitrogen excretions of the 9 groups of rats observed. The decreasing urinary nitrogen

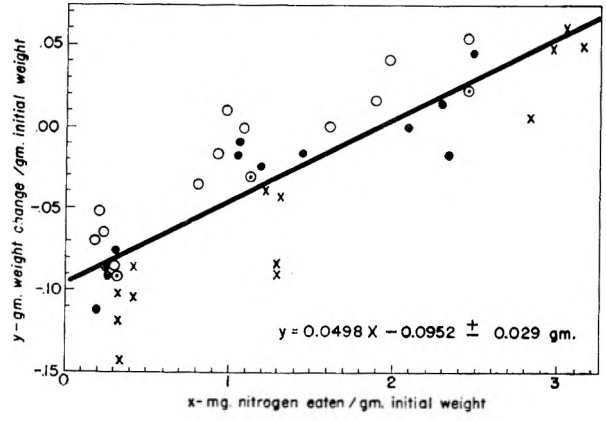
for all groups of animals in experiment I and for groups 5, 6, 7 and 8 in the other two experiments is similar to that observed by Burroughs et al. ('40) for rats on continuous low-nitrogen feeding and Bricker and Mitchell ('47) for rats on diets supplemented with 0 and 4% egg. Swanson and others ('44) observed a depression in urinary nitrogen when animals were changed from low-nitrogen diets to protein-supplemented diets. In our study, a reversed feeding sequence

TABLE 2

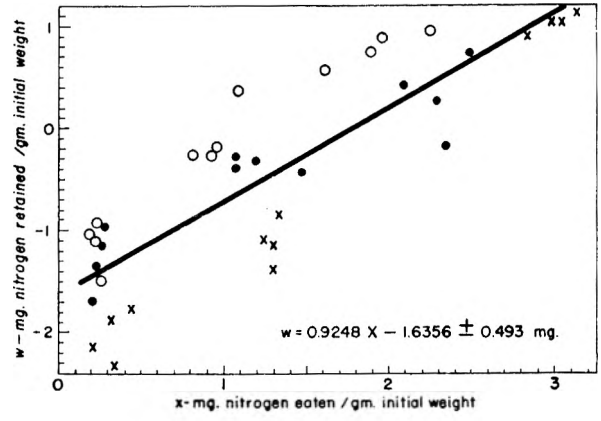
Diet sequence and mean urinary nitrogen

RAT GROUP	DEFATTED DRIED EGG IN DIET Percent			MEAN URINARY N mg/gm initial wt.		
	Period 1	Period 2	Period 3	Period 1	Period 2	Period 3
			Experiment I			
1	0	2	4	1.93	1.06	0.84
2	2	4	0	1.66	1.21	0.98
3	4	0	2	1.20	1.16	0.78
			Experiment II			
4	0	4	10	1.49	0.95	1.34
5	4	10	0	1.50	1.28	1.05
6	10	0	4	1.49	1.19	0.95
			Experiment III			
7	0	10	4	1.50	1.36	0.96
8	10	4	0	1.42	1.04	0.98
9	4	0	10	1.10	1.15	1.35

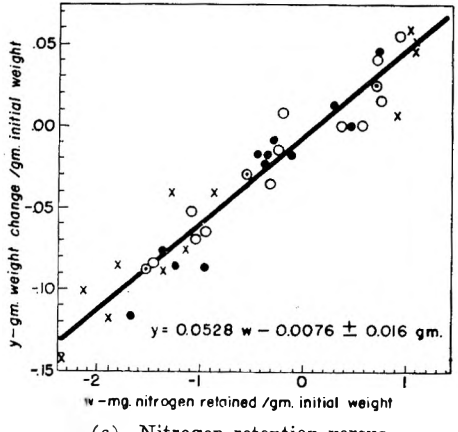
produced a similar depression in urinary nitrogen (groups 2, 3, 5, 6, 8, 9) and low-nitrogen diets resulted in the greatest nitrogen excretion only when fed first in a diet series (groups 1, 4, 7). The change from high to low urinary nitrogen for diets containing 0, 2 and 4% egg during the course of the experiment contrasts with the more constant urinary nitrogen for rats on the 10% egg diets. Thus rats on 0 to 4% egg diets attempt to adjust to the metabolic stress imposed by protein intakes which do not meet their "normal" protein requirements.



(a) Nitrogen intake versus weight change.



(b) Nitrogen intake versus nitrogen retention.



(c) Nitrogen retention versus weight change.

Fig. 1 Rat responses to 0, 2 and 4% egg diets in a cross-over type of experiment.

Responses obtained first, second and third in a feeding sequence are indicated by crosses (×), dots (●) and circles (○), respectively.

Variations in regressions used for predicting protein minima

Figure 1 shows scatter diagrams of the data used for predicting minimum nitrogen requirements for maintenance of body weight and nitrogen equilibrium in rats. These data were taken from experiment I. Regressions shown were computed using the formula for a straight line. Nitrogen intake versus weight change (fig. 1 a) and nitrogen intake versus nitrogen retention (fig. 1 b) show a marked tendency for points obtained from period two to fall between those for periods one and three, indicating that, for these rats, the sequence of feeding the diets might be a determining factor in predicting the minimum nitrogen intake required for maintenance of weight and nitrogen equilibrium. The protein stores of these rats apparently were undergoing depletion whenever either the 0 or 2% egg diets were fed, as evidenced by negative nitrogen balances for these two diets. Our results are similar to those reported by Rosenthal and Allison ('51) for dogs with different protein stores, in that differences in predicted requirements for the three feeding periods reflect differences in the protein stores of the animals.

Period effects do not appear to be a determining factor in predicting weight change from nitrogen retention (fig. 1 c).

Effects of experimental variables on rat responses to diets were estimated by grouping data from experiment I in 4 Latin squares for analysis of variance. Effects due to individual rats, groups of rats, feeding periods and diets were considered in the analysis. Only diet effects were found to be significantly different from the error sums of squares and these differences were highly significant ($P < 0.01$) for nitrogen intake, nitrogen balance, caloric intake and the weight change of the rats.

Interpretation of results from linear and multiple regressions

The interrelations of weight change, nitrogen intake, nitrogen retention and caloric consumption of rats on the 0, 2 and

4% egg diets were studied further by regression coefficients, correlation coefficients and standard errors of estimate of several linear and multiple regressions involving these variables. These are shown in table 3. Correlation coefficients for these regressions were all highly significant ($P < 0.01$).

For the linear regressions in which weight change is predicted nitrogen retention (equation 2) leads to a lower standard error of estimate than either nitrogen intake (equation 1)

TABLE 3

Linear and multiple regressions based on rat responses to 0, 2 and 4% egg diets

	CALCULATED REGRESSIONS [1]	STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT
		gm/gm I.W.	
(1)	$y = -0.0952 + 0.0498x$	0.029	0.85
(2)	$y = -0.0076 + 0.0528w$	0.016	0.96
(3)	$y = -0.1174 + 0.0979z$	0.041	0.66
(4)	$y = -0.0144 + 0.0042x + 0.0494w$	0.015	0.96
(5)	$y = -0.0408 + 0.0678x - 0.0519z$	0.027	0.87
(6)	$y = -0.0261 + 0.0500w - 0.0116z$	0.015	0.96
(7)	$y = -0.0292 - 0.0057x + 0.0528w + 0.0194z$	0.015	
		mg/gm I.W.	
(8)	$w = -1.6356 + 0.9248x$	0.493	0.87
(9)	$w = -3.0263 + 1.7269z$	0.760	0.64
(10)	$w = -0.2214 + 1.3932x - 1.3503z$	0.431	0.90

[1] y = gm wt. change/gm initial weight (I.W.).
x = mg N eaten/gm initial weight.
w = mg N balance/gm initial weight.
z = calories eaten/gm initial weight.

or caloric intake (equation 3). The standard error of estimate for weight change is not lowered in multiple regressions (equations 4 to 7). While the regression coefficient for nitrogen retention (w) remains almost constant throughout this series of regressions, those for nitrogen intake (x) and caloric intake (z) vary considerably. From these observations, it is concluded that weight change in the area of predominately negative nitrogen balance is more closely related to the ability of individual rats to retain nitrogen than to either the nitrogen intake or caloric intake of the rat.

From equations 8, 9 and 10 it must be concluded that in the area of negative nitrogen balance, nitrogen retention (w) is more dependent on nitrogen intake (x) than on caloric intake (z) since, in this series of regressions, nitrogen intake has a more constant regression coefficient and gives a lower standard error of estimate than caloric intake.

TABLE 4

Reproducibility of linear regressions

EGG IN DIETS	CALCULATED REGRESSIONS [1]	PREDICTED MINIMUM N REQUIREMENT
%		*mg/gm initial weight*
0, 2, 4	$y = 0.0498x - 0.0925$	2.33
	$y = 0.0528w - 0.0076$	0.16
	$w = 0.9248x - 1.6356$	1.77
0, 4	$y = 0.0508x - 0.0955$	1.91
	$y = 0.0527w - 0.0086$	0.14
	$w = 0.9693x - 1.6563$	1.71
0, 4 [2]	$y = 0.0439x - 0.0830$	1.89
	$y = 0.0434w - 0.0129$	0.30
	$w = 1.0129x - 1.6132$	1.59

[1] y = gm wt. change/gm initial weight.
x = mg N eaten/gm initial weight.
w = mg N retained/gm initial weight.
[2] Plus 8% fat.

Reproducibility of linear regressions

The linear regressions in table 3 were based on experiment I which resulted in a net weight loss and negative nitrogen balance for all rats over the 33-day experimental period. In table 4 these regressions are compared with several from experiment II. In this experiment, a 10% egg diet replaced the 2% egg diet in the feeding series. This change resulted in net weight gains and positive nitrogen balances for the 33-day period. These two sets of regressions are similar and give comparable minimum nitrogen requirement values.

Experiments II and III (table 4) were conducted simultaneously and are compared here to show the effect of adding

fat to the diet on predicted minimum requirements (experiment III). The regressions expressing the relationship between weight change and nitrogen intake indicate that the rats on the higher calorie diets required as great a nitrogen intake for weight maintenance (1.89 mg) as did animals on the low calorie diets (1.91 mg). The regressions for nitrogen intake versus nitrogen retention indicate that rats on the high calorie diets required less nitrogen for maintenance of nitrogen equilibrium (1.58 mg) than did rats on the low calorie diets (1.81 mg). This is in agreement with the finding that nonprotein calories have a sparing action on nitrogen in metabolic processes. Regressions showing the relationships between weight change and nitrogen retention indicate that animals on the higher calorie diets were in greater positive nitrogen balance (0.30 mg) at weight maintenance than were animals on the low calorie diets (0.14 mg). Thus added calories appeared to lead to greater nitrogen retention in these rats without having a similar "sparing effect" on body weight.

Regressions above and below maintenance intakes of nitrogen

Figure 2 shows regressions based on responses of rats to the diets which contained 0 and 4, and 4 and 10% egg and 4% lard. The nitrogen requirement for weight maintenance is again slightly higher than that for maintenance of nitrogen equilibrium. The curves for weight change and nitrogen retention are almost parallel below and in the area of maintenance. The small change in the direction of the nitrogen retention curve in the maintenance area indicates that nitrogen from the 10% egg diet is utilized almost as efficiently as that from the 2 and 4% diets. Thus, while the 4% egg diet appears to be completely adequate for maintaining weight and nitrogen equilibrium in these rats, there must be some other equally important demand for dietary nitrogen in the area above maintenance. It is questionable whether it is safe to assume that maintenance requirements for nitrogen

express actual needs of a healthy rat population, and further investigations of rat responses to protein intakes which result in a less efficient utilization of nitrogen are recommended. These findings suggest that it may be advisable to base recommendations for protein allowances on regression curves in the area of diminishing efficiency of utilization of nitrogen

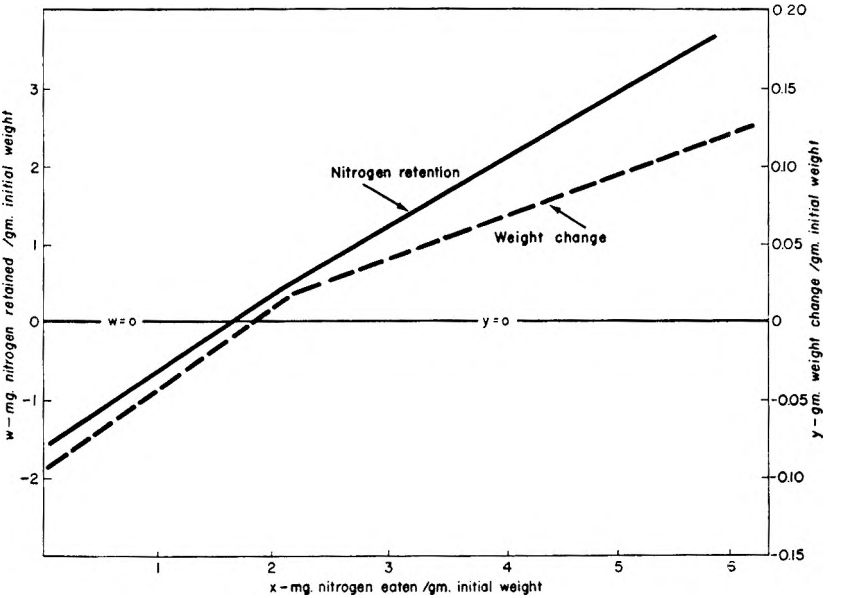


Fig. 2 Relation of nitrogen intake to weight change in rats fed 0, 2 and 4, and 4 and 10% egg diets.

rather than in the area of minimum maintenance requirements.

SUMMARY AND CONCLUSIONS

Data for predicting protein minima for 150-gm albino rats were obtained from feeding diets with 0 to 10% dried egg and 4 or 12% lard in a cross-over type of experiment.

Adjustment to the metabolic stress imposed by protein intakes which did not meet normal protein requirements was reflected in diminished nitrogen excretion of rats on diets containing 0 to 4% dried whole egg.

Scatter diagrams of data used for predicting minimum nitrogen intakes for maintenance of nitrogen equilibrium and body weight showed that points obtained in the second period of a cross-over experiment fell between those obtained in the first and third periods. This occurred regardless of the sequence in which the diets were fed. Thus, predicted minimum protein requirements of the rats reflected changes in protein stores during the course of the experiment.

Linear and multiple regressions of caloric intake, nitrogen intake, nitrogen retention and weight change indicated that for these rats, weight changes in the area of predominately negative nitrogen balance were dependent on the ability of individual rats to retain nitrogen on a given intake, and nitrogen retention in this area appeared to depend on nitrogen intake.

Linear regressions for predicting protein minima were reproduced in parallel experiments but were altered by varying the caloric value of diets.

A 4% egg diet was completely adequate for maintaining weight and nitrogen equilibrium in the rats observed; however, nitrogen utilization was almost as efficient in the area above maintenance as in that below maintenance.

It is recommended that the problem of estimating protein requirements from regression curves in the area of diminishing efficiency of utilization be investigated further.

ACKNOWLEDGMENT

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