THE ROLE OF MYO-INOSITOL IN PURIFIED DIETS [1]

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The role of inositol in plant and animal metabolism has been reviewed by Weidlein ('51). Experimental work from several laboratories has demonstrated that inositol is not required for optimum growth of rats on the usual purified diets in which casein provides the protein component (Ershoff, '44, '46; Jukes, '40; Richardson et al., '41; Ershoff and McWilliams, '43). However, several groups of investigators (Rose et al., '48; Ramasarma et al., '49; Kligler and Krehl, '52; Maddy and Swift, '52) have commonly included inositol as a constituent of experimental rations in which the nitrogen source consists of purified amino acids.

The nutritional studies reported here were designed to determine whether inositol has any beneficial effect on rats fed a diet composed of purified amino acids, oil, salts, purified vitamins and sucrose.

EXPERIMENTAL

Weanling male albino rats of the Harlan strain,[2] weighing 35 to 45 gm, were placed on laboratory stock rations for two days. They were then divided into two groups of 14 each, evenly distributed with respect to body weight and litters. The animals were housed singly in metabolism cages. One group was fed a basal diet and the other group received

[1] The word inositol as used in this paper refers to the common myo-inositol.
[2] Harlan Small Animal Industries, Cumberland, Indiana.

the basal diet plus inositol. After a two-day adjustment period on the test diets, the animals were found to range from 36 to 56 gm in weight. The rations and distilled water were supplied ad libitum. Weights of the rats and of the food consumed were recorded daily. The excreta were collected daily and were combined to form pools representing 7-day periods.[3] The urine was stored under toluene and was assayed for free inositol by the method described by Johnson ('49). The feces were stored under 10 *N* sulfuric acid and were assayed for total inositol after autoclaving in 10 *N* H_2SO_4 at 17 to 18 pounds of pressure for two hours.

Subsequently a third group of 7 weanling rats was given a casein-containing diet, but only the food consumption, weekly weight and carcass inositol content were determined.

At the end of 16 weeks the rats were sacrificed and their viscera were examined. The heart, one kidney and a portion of the liver were removed for microscopic examination. The carcass was immediately frozen, either in liquid nitrogen or in a dry ice acetone bath, and stored in a frozen state.

For digestion the thawed whole carcass was ground in a meat grinder, stirred in a Waring Blendor with 18% HCl (1 l per 400-gm rat), and refluxed overnight. The resulting material was filtered and assayed for inositol.

Composition of diets and vitamin supplements

Three diets were used: (1) the basal diet; (2) the basal plus inositol diet, prepared by mixing 10 mg of inositol with 100 gm of basal diet; and (3) a diet containing 19% of casein instead of the amino acids of the basal diet. The sodium phosphate was omitted and the sucrose content was adjusted to allow for these changes. The last diet was used for comparison of the growth of rats on the amino acid diets with that of animals on a more usual type of diet. All diets were prepared every 10 to 14 days and the stock was stored under refrigeration between feedings.

[3] The urine and feces from 6 rats in each group were used for this analysis.

The basal diet was essentially that described by Ramasarma et al. ('49; ration 9, p. 186) with the following exceptions: (1) L-lysine monohydrochloride was used at a level of 1.25 gm per 100 gm rations; and (2) the salt mixture found

TABLE 1

Vitamin supplements[1]

VITAMIN	AMOUNT PER 100 GM RATION
Thiamine hydrochloride, mg	0.50
Riboflavin, mg	1.0
Pyridoxine hydrochloride, mg	0.50
Nicotinic acid, mg	1 50
p-Aminobenzoic acid, mg	10 00
Calcium pantothenate, mg	2 50
Pteroylglutamic acid, μg	50.00
Biotin, μg	20.00
2-Methyl-1,4-naphthoquinone, mg	0.50
	AVE. DAILY INTAKE
Vitamin A[2] (as synthetic ester), I.U.	400
Vitamin D,[2] I.U. (as irradiated ergosterol, 400,000 U./gm)	10
Alpha-tocopherol,[2] Merck synthetic, mg	0.70
Vitamin B_{12},[3] μg	0.30

[1] Choline chloride was added to the final diet at a level of 100 mg per 100 gm of ration.

[2] Provided by oral administration twice weekly of 0.05 ml of a dilution of these materials in corn oil.

[3] Provided by oral administration twice weekly of "Betalin 12 Crystalline" (vitamin B_{12} crystalline, Lilly), 15 μg per milliliter.

in the U.S.P. XIV was used. Vitamin supplements were administered as specified in table 1. The amino acids were obtained from a commercial source[4] and were used without further treatment.

In order to check the possibility that the dietary fat might contain inositol as an inositol phosphatide, the corn oil[5] used in these rations was decomposed with sodium peroxide and

[4] Nutritional Biochemicals Corporation.

[5] Mazola.

zinc oxide, and was tested for the presence of organic phosphorus by the method of Fiske and Subbarow ('25). The phosphorus content was only 0.00015%. The phosphorus may be present as lecithin or cephalin, which are devoid of inositol. However, assuming that the phosphorus is present as an inositol phosphatide and the P-inositol ratio is 1:5, as suggested by Wooley ('43), only trace amounts of inositol were present in the corn oil.

The amino acids were thoroughly mixed in the proper proportions by use of a small ball mill, and then were combined with the balance of the ingredients to complete the rations. Nitrogen assays on the amino acid mixtures and on each lot of completed rations were performed to check the thoroughness of the mixing process.

The weight gains, protein efficiency and inositol content of the carcasses were ascertained and the data were treated statistically. The student "t" was used ($P = 0.05$) in order to determine the significance of the differences between the means.

DISCUSSION AND RESULTS

The weight gains and protein efficiency values for the three diets are recorded in table 2. No significant differences in average weight gain and protein efficiency were observed between the group of rats receiving the basal diet and those receiving the basal diet plus inositol. Moreover, there were no significant differences in average weight gains between these two groups of rats and the group that received the 19% casein diet. This latter group originally consisted of 7 animals; however, before the end of the test period, three of these rats developed severe dental malocclusion and failed to grow consistently. For this reason only 4 animals of this group are included in the final results.

The average weight gains (table 2) compare well with those reported by others using similar purified diets (Rose et al., '48; Ramasarma et al., '49). The protein efficiency of 2.53 following administration of the basal diet for 4 weeks com-

pares favorably with that (2.50) reported by Ramasarma et al. ('49) for a diet containing added inositol. From these data it appears that the addition of inositol to the usual amino acid basal ration did not affect the growth rate or efficiency of food utilization over a relatively long period of time.

TABLE 2

Weight increases and protein efficiency values

TREATMENT	NO. RATS IN GROUP	TIME		
		4 Wk.	40 Days	16 Wk.
		Weight increases		
Basal diet	13	119.8 ± 4.80 [1]	179.8 ± 8.42	316.3 ± 17.22
"Basal plus inositol" diet	13	121.2 ± 4.01	175.2 ± 6.59	302.5 ± 15.69
19% Casein diet	4	140.0 ± 16.39	209.3 [2] ± 21.23	324.8 ± 19.65
		Protein efficiency values		
Basal diet	13	2.53 ± 0.045	2.39 ± 0.064	1.34 ± 0.038
"Basal plus inositol" diet	13	2.46 ± 0.058	2.32 ± 0.051	1.29 ± 0.039
19% Casein diet	4	2.67 ± 0.137	2.32 [2] ± 0.085	1.22 ± 0.050

[1] Standard error.
[2] Value for 42 days.

TABLE 3

Urinary, fecal and carcass inositol values

DIET	URINE INOSITOL Range, mg per week	FECAL INOSITOL Range, mg per week	CARCASS INOSITOL Mean, μg per gram
Basal 12 animals	0.2–2.5	0.12–0.87	233
"Basal plus inositol" 9 animals	0.3–2.2	0.16–1.0	256
19% Casein 4 animals	..	..	222

The inositol content of the excreta and of the carcasses of the rats is shown in table 3. The urinary excretion of free inositol by the animals on the basal diet gradually increased from 202 μg during the first week to a maximum of approximately 2.5 mg per week at the 9th and 10th weeks, and remained constant thereafter. The range from the 9th to the 15th week was 1.97 to 2.9 mg excreted per week. The excretion of inositol by animals on the basal diet plus inositol was 318 μg for the first week; thereafter it increased steadily and became fairly constant at the 9th to 10th weeks at a level of 2.9 mg per week. The maximum range was 1.4 to 2.95 mg per week. There appeared to be no difference in the urinary output of inositol by the two groups of animals.

The fecal excretion of inositol as determined microbiologically after acid hydrolysis was rather erratic. It varied from 121 to 870 μg per week in rats on the basal diet and from 157 to 1,000 μg per week in animals on the basal diet plus inositol. There was no real difference in the rate of fecal excretion of inositol in the two groups of animals.

The inositol content of the carcasses of the three groups was similar. Individual values for the animals varied but little; apparently the tissue content of inositol is not dependent upon the dietary intake of this material.

One of the animals on the basal diet had an enlarged, hypertrophic heart, a pale liver, and enlarged kidneys. Microscopically the kidney showed severe glomerulonephritis. No explanation for this condition could be found. All other animals were free from gross pathologic changes.

Microscopic examination showed a small amount of fat in the livers of some of the animals on all three diets. In addition, two of the animals on the basal diet had a slight focal myocarditis and one had a slight pericarditis. One of the animals on the inositol-containing diet also had a slight focal myocarditis. There were no significant differences in the gross and microscopic appearance of the viscera of the three groups of animals.

Although the basal diet contained no free inositol as determined microbiologically, after hydrolysis with 6 N H_2SO_4 for 4 hours a small amount of inositol was found. This level (4 to 5 mg per 100 gm diet) was one-half the quantity added to the diet. A comparable hydrolysis of the sucrose used indicated that it was probably the source of this "bound inositol." If this was actually inositol, the quantity present would make its isolation from the hydrolysis mixture difficult. The form of "bound inositol" does not appear to be associated with phosphorus, as no phosphorus could be found in the ash from the sucrose. However, the possibility that trace amounts of a compound of the type o-α-d-galactopyranosyl-myo-inositol (Brown and Serra, '52) were present could not be eliminated.

SUMMARY

The addition of inositol to a purified diet consisting of amino acids, sucrose, corn oil, salts and vitamins did not affect the growth rate of the Harlan strain white rat. Gross and microscopic examination of the animals and tissues did not show any differences between the two groups. In addition it was shown that this purified diet was adequate for good growth of these rats over a 16-week period.

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EXPERIMENTAL DENTAL CARIES

V. THE EFFECTS OF DESALIVATION AND CASTRATION ON CARIES AND FLUORINE STORAGE IN THE RAT [1]

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ONE FIGURE

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INTRODUCTION

The effects of modified salivary function on dental caries experience in rats have been reviewed (Muhler and Shafer, '53), as have the effects of castration (Muhler and Shafer, '52). However, previous workers have not observed the combined effect of castration and desalivation in the same animals on the incidence of dental caries. Therefore, the purpose of this paper is to report data on the degree of dental caries in rats which have been castrated, desalivated, or castrated and desalivated, in comparison to the degree in control animals, as well as to investigate the storage of fluorine in each group.

PROCEDURE

Approximately 150 animals [5] were divided into 4 experimental groups at 25 to 27 days of age, so that each group was about equal as to sex and weight. All animals were

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[4] School of Dentistry.

[5] Sprague-Dawley strain, Carl Wilson, Beach Grove, Indiana.

housed in an air-conditioned room and were kept in cages with raised screen bottoms. One group was desalivated, another castrated and a third both desalivated and castrated, placed on our cariogenic diet (Muhler and Day, '50) with a dietary fluorine concentration of 0.3 p.p.m. and given distilled water at 30 days of age. A 4th group was not operated on, received the same diet and served as controls. In the first experiment all the food and water was available ad libitum. The duration of the experiment was 140 days, after which time the animals were sacrificed by ether inhalation and the heads and femurs saved for analysis (Muhler, Nebergall and Day, '53).

In these experiments desalivation consisted of complete surgical removal of the submaxillary and sublingual glands. The greater portion of the parotid was also removed but, due to the gland's diffuseness, complete removal was not probable.

A one-half-inch ventral mid-line incision was made, exposing the superficial fascia containing the salivary glands. By blunt dissection the overlying fascia was sectioned and the three pairs of glands were separated into two lateral halves. One portion was then lifted ventrally and laterally. In this extended position the 5 principal vessels were tied off. Anteriorly, the lingual artery and the two branches of the posterior facial vein were tied off with one suture of surgical thread. Posteriorly, the posterior auricular artery and vein were tied off with one suture. By maintaining the glands in the extended position and cutting through the supporting fascia along the ventral surface of the external jugular vein, the salivary tissues were easily removed. The procedure was then duplicated for the contralateral glands.

DATA AND DISCUSSION

The data pertaining to the incidence of dental caries in the first experiment are given in table 1. There was a significant reduction in caries in the orchiectomized and ovariectomized

animals. Previous experience of the authors (Muhler and Shafer, '52) had indicated that orchiectomy consistently results in reduction in caries but the response to ovariectomy was less definite. However, in the experiments reported here the animals were operated on and placed on the cariogenic diet 10 days to two weeks earlier than in previous work. Thus, the age at which the animals were operated on could have accounted in part for these discrepancies.

TABLE 1

The effect of desalivation, castration and combined desalivation-castration on the dental caries experience in rats

GROUP	SEX	NUMBER OF ANIMALS	NUMBER OF LESIONS	EXTENT[1]	CARIES CHANGE
					%
Control	Male	19	8.2	2.14	..
	Female	19	7.5	2.23	..
Castrate	Male	17	5.5	1.93	— 39
	Female	15	4.7	1.92	— 37
Desalivated	Male	17	12.9	2.54	+ 55
	Female	15	12.6	2.60	+ 68
Desalivated-castrated	Male	16	10.1	2.50	+ 20
	Female	15	9.9	2.44	+ 24

[1] Computed by estimating the degree of damage to the molar teeth of rats. If from zero to one-fourth of the tooth is destroyed, a value of one is assigned to this tooth; if from one-fourth to one-half is decayed, then a value of two is indicated, while if more than one-half is carious, the tooth is given a value of three. The arithmetic mean is then calculated for each group.

The effects of desalivation confirmed previous work (Muhler and Shafer, '52) and indicated about a 60% increase in caries in the combined sexes. The number of lesions was significantly greater and the severity (extent) of cavities was considerably greater than in the rats which were not operated on. In the group which was both desalivated and castrated, the cariogenic effect of desalivation was noticeably reduced. However, even though the number of lesions was considerably less in the combined operation group than in the desalivated

group, the severity of the lesions was quite similar. Thus, it may be that desalivation has little effect on the initiation of caries but a definite effect on their extension. The effect of the operations on the caries of the various groups is illustrated in figure 1.

The storage of fluorine and the amounts of calcium and phosphorus in the femurs of the various experimental groups are given in table 2. These results make it appear that

TABLE 2

Body weight increase, fluorine storage and calcium and phosphorus content of the femurs

(Dietary fluorine content, 0.3 p.p.m.)

GROUP	FINAL BODY WEIGHT	WEIGHT INCREASE	ASHED FEMURS					
			Weight	Ash	F conc.	Total F	Ca conc.	P conc.
	gm	*gm*	*gm*	*%*	*μg/gm*	*mg*	*mg %*	*mg %*
				Males				
Control	320	246	0.3666	61.5	73.9	0.032	40.6	19.4
Desalivated	283	212	0.2861	55.1	44.5	0.011	41.9	19.2
Castrated	284	214	0.3066	56.5	48.3	0.014	40.6	19.6
Desalivated-castrated	245	168	0.2608	54.3	39.9	0.010	43.2	18.9
				Females				
Control	212	143	0.2557	57.2	101.2	0.026	41.9	19.5
Desalivated	199	127	0.2243	56.8	85.5	0.021	43.6	19.2
Castrated	254	181	0.2586	54.0	73.3	0.019	42.7	19.1
Desalivated-castrated	251	180	0.2449	59.8	41.3	0.010	40.8	19.2

both desalivation and castration influence the amount of fluorine retained by the skeleton, with the effect of the combined desalivation-castration operation being the most pronounced. This is in line with previous work (Muhler and Shafer, '53) based on the use of a different diet, in which it was shown that desalivated animals store less fluorine than controls under ad libitum feeding conditions. Castrated males appeared to store slightly more fluorine than desalivated males, even though the final body weights of the two groups

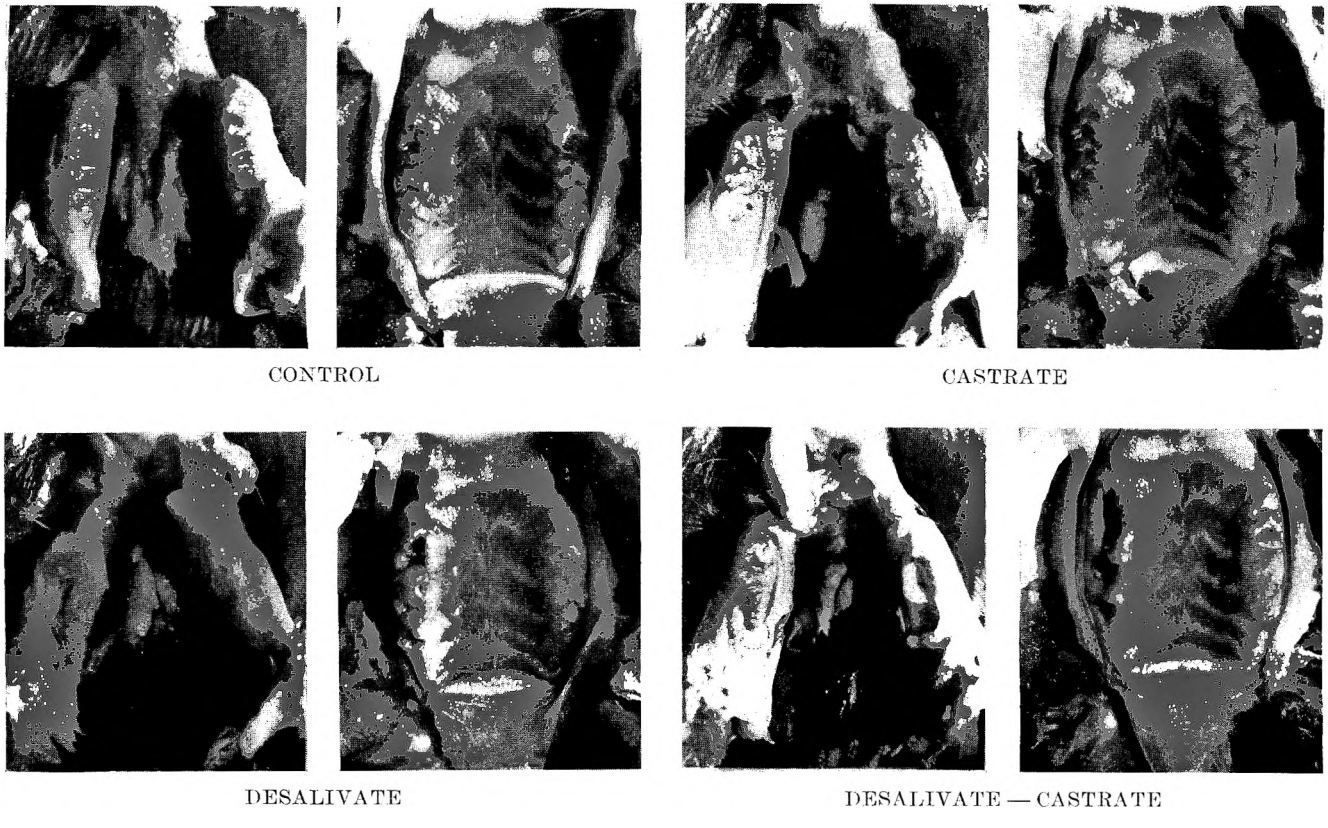

Fig. 1. A comparison of the dental caries incidence in the various experimental groups. These illustrations were selected from animals having the mean number of carious teeth in each group.

were identical. However, this did not hold true for the females, since the ovariectomized animals stored slightly less fluorine than the desalivated females. The differences were so small in both cases that they may not be significant.

It is possible that the decreased fluorine storage in the groups operated on was a direct result of ingesting less food. This is logical, since the male controls were larger animals than any of the three groups operated on. However, the combined effects of desalivation and castration seemed to eliminate the differences in fluorine storage between the males and females. Further obvious discrepancies in postulating a rule suggesting a direct relationship between food ingestion and fluorine storage, regardless of the sex of the animal, are apparent in the data on the control females and the ovariectomized rats. The fluorine concentration in the castrated females was about 30% less than in the controls, while the latter animals were about 20% larger. Likewise, a comparison of the castrated and castrated-desalivated females shows that the final body weight and weight gain were the same and yet the latter group stored considerably less fluorine.

Also, desalivation resulted in a somewhat decreased body weight in the males, but in the female group this effect did not appear to be as pronounced. Furthermore, previous work from these laboratories has indicated that desalivated animals eat only about 1 gm of food per day less than unrestricted control animals, so it seemed improbable that the differences in fluorine storage were due entirely to differences in the amount of food ingested. Also, it has been found (Bixler, Muhler and Shafer, '53) that desalivated animals gain less weight on the same amount of ingested food. Thus, it appears that there may have been some growth-producing or food-assimilating factor lacking in the desalivated animals that accounts for both the failure to gain weight normally and the change in the storage of fluorine. It is inviting to consider that the castrated animals appeared to be similar to the de-

salivated animals, because castration affects the salivary glands (Shafer and Muhler, '53).

In order to investigate further the effect of food ingestion on fluorine storage in desalivated animals, a paired-feeding experiment was conducted. In this experiment the same amount of food eaten (fluorine content of diet was 0.6 p.p.m.) by the desalivated animals was given to the controls. The results are given in table 3, and indicate that fluorine storage

TABLE 3

A comparison of body weight, food ingestion and fluorine storage in desalivated rats pair-fed to controls not operated on

(Dietary fluorine content, 0.6 p.p.m.)

GROUP	NUMBER OF ANIMALS	FINAL BODY WEIGHT	WEIGHT GAIN	FOOD CONSUMPTION PER ANIMAL PER DAY	ASHED FEMUR ANALYSIS		
					Weight	F conc.	Total F
		gm	*gm*	*gm*	*gm*	*μg/gm*	*gm*
				Males			
Control	18	308	231	13.0	0.2894	133	0.039
Desalivated	18	273	212	12.9	0.2855	128	0.037
				Females			
Control	17	207	139	11.2	0.2406	149	0.034
Desalivated	17	196	131	11.3	0.2244	155	0.035

under paired feeding conditions eliminates the differences between the two groups found with respect to the ad libitum group. Although the two groups ingested the same amount of food, the desalivated animals again failed to gain weight, as well as the controls, indicating that their utilization of food may not have been as efficient. As with the males, the desalivated females attained a slightly smaller final body weight than the controls on the same food intake.

These combined data indicate that the storage of fluorine in the two sexes is quite dependent upon the food intake as well as the rate of growth. In the paired feeding experiment, the weight of the desalivated females was somewhat

less than that of the controls, but the fluorine storage was almost identical. These findings indicate, then, that the results of the ad libitum feeding experiment were caused by the desalivated groups' receiving less fluorine, through the ingestion of less food. However, the findings relevant to the female castrated and desalivated-castrated group must be explained in some other way, since they were decidedly larger animals but stored considerably less fluorine. These experiments serve to emphasize the point that mineral metabolism is greatly influenced not only by the amount of food ingested but also by the growth process as manifested by the assimilation and utilization of food, as well as by the influences that the endocrine system may exert in the metabolism of food and food products.

SUMMARY

The effects of desalivation and castration on dental caries and the storage of fluorine in the skeletal system are reported. Desalivation resulted in a marked increase in the incidence and severity of dental caries. Castration, on the other hand, in both sexes reduced caries development significantly. A sex difference in the storage of fluorine was noted, with females storing higher amounts than males. Fluorine storage under ad libitum feeding conditions indicated less fluorine in both males and females in the skeletons of the desalivated, castrated or combined desalivated-castrated groups, but under paired-feeding conditions the differences in fluorine storage between the desalivated and control groups disappeared. Fluorine storage in desalivated animals appears to be related to body growth and food ingestion in both males and females, but the fluorine storage data for a castrated and for a combined desalivated-castrated female group indicate that additional information is needed.

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ESSENTIAL FATTY ACIDS AND HUMAN NUTRITION

I. SERUM LEVEL FOR UNSATURATED FATTY ACIDS IN HEALTHY CHILDREN [1]

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TWO FIGURES

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Blood serum levels of dienoic, trienoic and tetraenoic acids in dogs have been found to reflect the nutritional status of animals maintained on varying amounts and kinds of fat (Wiese and Hansen, '51, '53). The values for these unsaturated fatty acids, expressed as per cent of the total fatty acids, correlated well with the dietary history and physical appearance of the animals as well as with the histologic structure of the skin. On the basis of these findings, it appeared feasible to use the serum level of linoleic and arachidonic acids as a means of evaluating the nutritional significance of these fatty acids in children. First, however, it was necessary to establish the serum level of unsaturated fatty acids in healthy children, particularly in relation to the dietary intake of specific fatty acids. The results of such a study are presented in this report.

MATERIAL AND METHODS

The semimicrospectrographic procedure of Wiese and Hansen ('53) was used to determine the level of unsaturated fatty

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

acids in the serum after a 12- to 15-hour fast for a total of 93 human subjects who were considered to be in a good state of nutrition. The subjects fell in three groups. Group I was composed of 60 children from two orphanages. The majority of these children were under observation about one year, during which time a careful clinical evaluation of their nutritional status was made and institutional dietary surveys were conducted. The age range was from 4 to 15 years. Group II consisted of 26 hospitalized children who were in a good state of nutrition from the clinical viewpoint and were given the regular hospital diet. The age range was from two to 15 years. Group III was composed of 15 infants and children from 6 months to 4 years of age. Eight of the children were those under 4 years of age in groups I and II and the remaining 7 were healthy infants and young children of staff physicians.

RESULTS

Group I — children from orphanages G and L

Clinical estimation of nutritional status. Repeated physical examinations were made on 60 children independently by two staff pediatricians. Special attention was given to the condition of the skin, hair, eyes, mouth, tongue and posture, to signs of infection and to degree of vigor and vitality. The height and weight of each child was charted. The majority of the children fell within the 10 to 90 percentile, using Stuart's tables from Boston as a standard. The developmental level was plotted on the Wetzel grid and the distribution of the auxodromes was found to be satisfactory. These are presented in figure 1. There were 7 children who could not be used in the statistical consideration, inasmuch as one child was a chondrodystrophic dwarf, another (a half sibling) was abnormally short and 5 others were members of a family which was characterized by smallness of stature. However, these 7 abnormally small subjects were in good condition nutritionally.

Laboratory evidences of nutritional adequacy. To support clinical evidence of nutritional adequacy, laboratory studies including total serum proteins with albumin, globulin fractions, hemoglobin and erythrocyte counts were made. In order to discover evidence of obscure infection, leukocyte and differential counts were made. All these values were within normal limits. A summary of these data for the two orphanages is given in table 1.

The results of the clinical studies were remarkably uniform and justified the term "well-nourished child" in every instance.

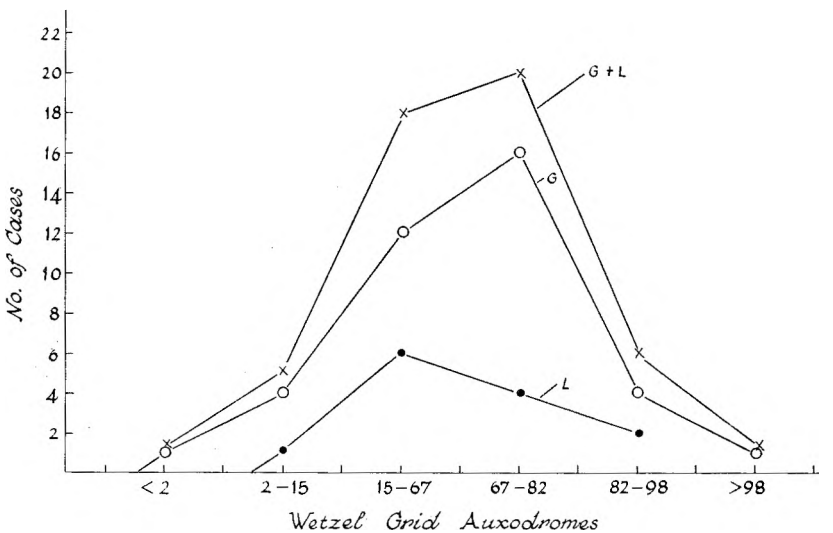

Fig. 1 Wetzel grid auxodromes for well-nourished control children.

TABLE 1

Mean and standard deviations for serum proteins and blood counts on 60 well-nourished children 4 to 15 years of age

SERUM PROTEINS		BLOOD COUNTS		DIFFERENTIALS	
	gm %				%
Total	7.04 ± 0.85	Hgb	13.2 ± 1.53 gm %	PMN	63.9 ± 3.6
Albumin	4.88 ± 0.46	RBC	4.46 ± 0.36 millions/mm³	Stab.	1.6 ± 1.8
Globulin	2.16 ± 0.55	WBC	7,631 ± 1,320/mm³	Lympho.	32.4 ± 0.4
				Eosino.	2.0 ± 1.8

Dietary history. Institutional dietary surveys of the two orphanages were made by our hospital dietitians. Average daily food consumptions were ascertained for 4 consecutive days. In orphanage G, the average daily caloric intake was estimated to be 1,790 calories, with 14% from protein, 38% from fat and 48% from carbohydrate. The dietary fats were milk, egg, meat, including ham and bacon, margarine and a vegetable shortening. From analyses of the fats, linoleic acid

TABLE 2

Summary of 102 determinations of serum fatty acid values for 60 well-nourished children from orphanages G and L

GROUP	AGE	TOTAL FATTY ACIDS	DIENOIC	TRIENOIC	TETRAENOIC	HEXAENOIC
			Per cent of total fatty acids			
	years	*mg %*				
Orphanage G, 40 children	4–15					
Mean		296	30.4	1.4	10.0	4.7
SD		43.6	3.05	0.61	1.32	1.47
SE_m		5.5	0.38	0.08	0.16	0.18
Orphanage L, 20 children	4–13					
Mean		307	30.2	1.6	10.5	3.4
SD		38.4	1.48	0.74	1.72	1.22
SE_m		6.2	0.24	0.12	0.27	0.19
P [1]		0.19	0.66	0.17	0.11	< 0.001

[1] Derived from Student's "t" test.

constituted 3.2% of the total calories. There were only trace amounts (less than 0.5%) of arachidonic acid in a few of the fats. In orphanage L, the average daily caloric intake was somewhat less, 1,493 calories, with 16% from protein, 35% from fat and 49% from carbohydrate. The dietary fats were milk, egg, meat, bacon, margarine and vegetable shortenings. Analyses of these fats showed that linoleic acid constituted 3.6% of the total calories. Again, no or negligible amounts of arachidonic acid were found.

Distribution of unsaturated fatty acids in the serum. In table 2 is given a statistical summary of the distribution of

dienoic, trienoic, tetraenoic and hexaenoic acids in the total fatty acids of serum for children from the two orphanages. There were 102 determinations on the 60 children, blood samples being taken from the majority of the children at 6-month intervals. It will be noted there are no statistical differences in the amount of total fatty acids or in the serum level of the two, three and 4 double-bond fatty acids between

TABLE 3

Summary of serum fatty acid values for 60 well-nourished children from orphanages G and L and 26 well-nourished hospitalized children

GROUP	AGE	TOTAL FATTY ACIDS	DIENOIC	TRIENOIC	TETRAENOIC	HEXAENOIC
			Per cent of total fatty acids			
	years	*mg %*				
I	4–15					
Orphanages G and L						
Mean		301	30.3	1.5	10.2	4.2
SD		35.9	2.83	0.62	1.44	1.53
SE_m		3.59	0.28	0.06	0.14	0.15
II	2–15					
Hospitalized						
Mean		280	28.4	1.5	11.1	2.4
SD		49.8	4.58	0.71	2.48	1.41
SE_m		10.4	0.86	0.13	0.47	0.26
P [1]		0.06	0.04	1.0	0.07	< 0.001

[1] Derived from Student's "t" test.

the two groups of children. The mean values for these unsaturated fatty acids for all the children in the two orphanages may be taken, therefore, as reference standards for a control group of well-nourished children 4 to 15 years of age. Large variations were found in the hexaenoic acid content of the serum, and the mean values for the two groups were significantly different.

Group II — well-nourished, hospitalized children

This group of 26 children was composed of hospitalized patients who were considered to be in a good state of nutri-

tion and who were given the regular hospital diet. The diagnoses in these patients included asthma, amebiasis, cerebropalsy, psychoneurosis, mental deficiency, inactive rheumatic fever, early Hand-Schüller Christian disease and post-operative cases, together with patients recovering from burns and pneumonia. The hospital dietitian's records showed that fat constituted 42% of the total calories of the diet. The fats were derived from milk, egg, meat, margarine, vegetable oils

TABLE 4

Summary of serum fatty acid values for 60 well-nourished children 4 to 15 years old and 15 healthy children 6 months to 4 years of age

GROUP	AGE	TOTAL FATTY ACIDS	DIENOIC	TRIENOIC	TETRA-ENOIC	HEXA-ENOIC
			Per cent of total fatty acids			
	years	*mg %*				
I Control	4–15					
Mean		301	30.3	1.5	10.2	4.2
SD		35.9	2.83	0.62	1.44	1.52
SE_m		3.59	0.28	0.06	0.14	0.15
III Infants and small children	6 mo.–4					
Mean		283	27.5	1.3	9.3	3.1
SD		64.6	3.94	0.07	2.43	1.85
SE_m		15.6	0.98	0.01	0.59	0.44
P [1]		0.26	0.006	0.001	0.14	0.02

[1] Derived from Student's "t" test.

and a compound lard. Analyses of these fats showed that an average of 5.6% of the total calories was provided by linoleic acid. A small amount of arachidonic acid ($< 0.1\%$ of total calories) was present. A statistical summary of the distribution of the two, three, 4 and 6 double-bond fatty acids in the serum of these children, compared with that of the control group from orphanages G and L, is given in table 3. "P" values for hexaenoic acid were calculated separately for each orphanage and were found to show the same degree of

significance as for the combined orphanages. Although the mean values for the hospitalized children differed slightly from those for the control group of children from the orphanages, the differences were not significant except in the case of hexaenoic acid.

Group III — infants and children 6 months to 4 years of age

The serum level of unsaturated fatty acids in these 15 well-nourished infants and young children was compared with that of control group I in order to determine whether or not there is a relationship between age and the distribution of unsaturated fatty acids in the serum. A statistical summary of the findings is given in table 4. The mean values for infants and children under 4 years of age were less than for the control group 4 to 15 years of age.

DISCUSSION

It will be noted from tables 2, 3 and 4 that results are given for the serum levels of three and 6 double-bond fatty acids as well as for the essential fatty acids. Using the semimicrospectrographic method of Wiese and Hansen ('53) for determining the unsaturated fatty acid content of human serum, it was found that absorption for conjugated fatty acids occurred at 2,350, 2,700, 3,000, 3,475 and 3,750A. This indicates the presence in human serum of two, three, 4, 5 and 6 double-bond fatty acids. Since publication of the semimicrospectrographic method, a sample of the methyl ester of a 6 double-bond fatty acid of known composition (92.5% hexaenoic acid) was obtained [2] and the $E^{1\%}_{1\ cm}$ values for hexaenoic acid determined. The amount of pentaenoic acid could not be calculated, inasmuch as the necessary standard for this acid has not been available. Absorption coefficients used as stand-

[2] From the Hormel Institute, through the courtesy of Dr. Walter Lundberg.

ards for calculation of per cent of two, three, 4 and 6 double-bond fatty acids were as follows:

$E^{1\%}_{1\ cm}$ *for conjugated fatty acid soaps*

	2,350A	2,700A	3,000A	3,475A	3,750A
Dienoic	779				
Trienoic	524	518			
Tetraenoic	484	405	350		
Hexaenoic	469	307	218	185	98

Available absorption data would indicate that when the results given in tables 2, 3 and 4 are corrected for pentaenoic acid, the tetraenoic acid values reported will be decreased by approximately 1.5% of the total fatty acids, with no essential change in the dienoic and trienoic acid levels. It is felt, therefore, that with this small correction in mind, the blood levels found for the 60 institutional children may be used as reference standards for the essential fatty acids in the serum of healthy children 4 to 15 years of age. The relatively high serum levels for tetraenoic acid were maintained by these children on a dietary intake comprising about 3% of the total calories as linoleic acid and with only trace amounts of arachidonic acid ($< 0.1\%$). The data indicate that there is no dietary requirement for arachidonic acid in healthy children when linoleic acid is supplied in the diet. The minimum dietary requirement for linoleic acid to maintain normal blood levels for healthy children is yet to be determined. Essentially these same serum levels for linoleic acid and arachidonic acids were found for the group of 26 well-nourished hospitalized children, in whom the dietary intake of linoleic acid during the period of hospitalization was about 5% of the total calories.

Although the mean values for dienoic and tetraenoic acids in the serum for the control subjects in group I and the infants and children in group III did not show highly significant differences, when individual results for these two unsaturated fatty acids are correlated with the age of the subjects and plotted as scattergrams, there appears to be a gen-

eral tendency for the levels to be somewhat lower in infants than in children over two years of age (see fig. 2). Hence the mean values found for dienoic and tetraenoic acids in the 60 healthy children in orphanages G and L, where the age range was 4 to 15 years, may not be applicable as normal blood levels for well-nourished infants.

Fig. 2 Serum levels for dienoic and tetraenoic acids in relation to age of well-nourished children.

The very small amount of trienoic acid found in the serum of well-nourished children compared with the high serum levels of dienoic and tetraenoic acids may be of some importance in relation to nutritional status. In dogs suffering from fat deficiency, the trienoic acid content of the serum has consistently been found to be high, 16% of the total fatty acids (Wiese and Hansen, '53). As the gross and microscopic signs of fat deficiency disappear after the addition to

the diet of fat containing linoleic acid, the trienoic acid in the serum decreases to low levels, whereas the dienoic and tetraenoic acids increase in amount. A similar observation with respect to a high trienoic acid content of the heart of fat-deficient rats was observed by Rieckehoff, Holman and Burr ('49). After supplementation of the diet with corn oil, the amount of three double-bond fatty acid in the heart was reduced from 22.2 to 4.8%.

The significance of pentaenoic and hexaenoic acids in human serum cannot be evaluated adequately at present. They have not been found to be nutritionally significant in experimental animals, although some consideration has been given to them from a metabolic standpoint inasmuch as they have been identified in various animal tissues (Nunn and Smedley-MacLean, '38; Rieckehoff, Holman and Burr, '49; Widmer and Holman, '50; Holman and Taylor, '50; Reiser, '50, '51).

SUMMARY

The blood serum levels for dienoic, trienoic, tetraenoic and hexaenoic acids were determined on 93 well-nourished infants and children.

The serum levels for two, three and 4 double-bond fatty acids are in the same range for both well-nourished hospitalized children and healthy non-hospitalized children.

There appears to be a slightly lower level for dienoic and tetraenoic acids in the serum of infants than in children two to 15 years of age.

The trienoic acid content of the serum for healthy children is relatively low.

On a dietary intake comprising about 3% of the total calories as linoleic acid, the mean serum levels of two, three and 4 double-bond fatty acids for 60 healthy control children 4 to 15 years of age were, respectively, 30.3, 1.5 and 10.2% of the total fatty acids. On the basis of these blood levels, the data indicate that there is no dietary requirement for arachidonic acid for healthy children when linoleic acid is supplied.

The hexaenoic acid content of the serum of healthy children shows wide variations.

The significance of pentaenoic and hexaenoic acids in the serum of healthy children is not known.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Dr. G. David Ford in the clinical examinations and of Dr. J. Allan Scott and Miss Mary G. Westbrook in the statistical analyses.

The authors are indebted to Mrs. Peter Wong and Miss Joy Moore for the dietary surveys.

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ESSENTIAL FATTY ACIDS AND HUMAN NUTRITION

II. SERUM LEVEL FOR UNSATURATED FATTY ACIDS IN POORLY-NOURISHED INFANTS AND CHILDREN [1]

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ONE FIGURE

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From a limited number of studies with human beings (Brown et al., '38; Hansen and Wiese, '44; Hansen and Burr, '46; Hansen et al., '47), the data concerning blood lipids indicate that human subjects do not synthesize certain highly unsaturated fatty acids. It has been found further that healthy infants and children maintain relatively high serum levels for linoleic and arachidonic acids (Wiese, Gibbs and Hansen, '54). With a dietary intake comprising about 3% of the total calories as linoleic acid, the latter authors found the serum levels of dienoic and tetraenoic acids to be, respectively, 30.3 and 10.2% of the total fatty acids. In order to help in an evaluation of the relationship between these fatty acids and nutritional status, a study has been made of the blood values for the essential fatty acids in children showing varying states of nutrition. The results of this study are reported here.

MATERIAL AND METHODS

Two groups of hospitalized infants and children were studied. Group I was composed of 23 subjects 4 months to 13

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

years of age who were considered to be in a fair state of nutrition from the clinical viewpoint. The diets varied so greatly that no estimate of the intake of fat was attempted. Group II consisted of 34 malnourished infants and children, all of whom were markedly underweight and presented the typical withered, wrinkly skin and apathy of chronic malnutrition. In 4 instances there were associated with these signs symptoms which were comparable to those found in experimental fat-deficient puppies, namely, desquamation, erythematous patches, alopecia and in one case a history of chronically draining ears. The severe nutritional states were attributed to underfeeding, starvation following prolonged diarrhea, or inability to absorb fat normally, as in cystic fibrosis of the pancreas or the celiac syndrome. No estimate of the dietary intake was made. The age range was three months to 11 years. Many of the children were under two years of age.

The serum levels of two, three, 4 and 6 double-bond fatty acids were determined for each child after a 10- to 14-hour fast by a semimicromethod for measuring the unsaturated fatty acids of blood serum (Wiese and Hansen, '53).

RESULTS

Statistical summaries of the serum fatty acid data for the 24 fairly well-nourished children in group I and the 34 malnourished infants and children in group II, compared with those for the 60 control well-nourished children previously reported (Wiese, Gibbs and Hansen, '54), are given in tables 1 and 2. It will be noted that there were no significant differences in the amount of total fatty acid for either of the groups of inadequately nourished children. The serum levels of dienoic and tetraenoic acids were significantly less in the children considered clinically to be in a fair state of nutrition than in the control group (see table 1). The trienoic acid content of the serum in the fairly well-nourished children was higher than in the well-nourished group, although the difference was not highly significant. Hexaenoic acids showed

great individual variations, as in the case of control children, but mean values were significantly lower in group I than in the controls.

TABLE 1

Summary of serum fatty acid values for 60 well-nourished and 24 fairly well-nourished children

GROUP	AGE	TOTAL FATTY ACIDS	DIENOIC	TRIENOIC	TETRA-ENOIC	HEXAENOIC
			Per cent of total fatty acids			
	years	*mg %*				
Non-hospitalized	4–15					
Nutrition, good						
Mean		301	30.3	1.5	10.2	4.2
SD		35.9	2.83	0.62	1.44	1.53
SE_m		3.59	0.28	0.06	0.14	0.15
Hospitalized	4 mos.–13					
Nutrition, fair						
Mean		289	19.1	2.2	8.1	1.8
SD		76.1	3.30	1.08	2.62	1.00
SE_m		16.2	0.69	0.22	0.54	0.21
P [1]		0.47	< 0.001	0.002	< 0.001	< 0.001

[1] Derived from Student's "t" test.

TABLE 2

Summary of serum fatty acids for 60 well-nourished and 34 malnourished children

GROUP	AGE	TOTAL FATTY ACIDS	DIENOIC	TRIENOIC	TETRA-ENOIC	HEXAENOIC
			Per cent of total fatty acids			
	years	*mg %*				
Non-hospitalized	4–15					
Nutrition, good						
Mean		301	30.3	1.5	10.2	4.2
SD		35.9	2.83	0.62	1.44	1.53
SE_m		3.59	0.28	0.06	0.14	0.15
Hospitalized	3 mos.–11					
Nutrition, poor						
Mean		278	12.0	2.4	7.0	1.8
SD		59.3	4.69	1.20	2.21	1.37
SE_m		9.87	0.75	0.19	0.35	0.22
P [1]		0.03	< 0.001	< 0.001	< 0.001	< 0.001

[1] Derived from Student's "t" test.

In table 2 it will be noted that mean serum levels for all the unsaturated fatty acids in the malnourished infants and children show highly significant differences from those of the control group of institutional children. Again, the amounts of the two, 4 and 6 double-bond fatty acids were less and the amount of three double-bond acid was greater in the poorly-nourished group than in the control well-nourished children.

DISCUSSION

From the data presented in tables 1 and 2, it becomes apparent that the total amount of fatty acid in blood serum is not a valid indication of nutritional status. However, the serum levels of dienoic, tetraenoic and hexaenoic acids are significantly lower in malnourished than in well-nourished children. This indicates that poorly-nourished children who receive inadequate diets or are unable to absorb nutrients normally do not suffer from low blood fat levels but that the fat which they synthesize to maintain average levels is lacking in certain unsaturated fatty acids. Healthy children, on the other hand, who receive diets adequate to maintain them in good states of nutrition, retain large amounts of unsaturated fatty acids, as is indicated by high blood levels for certain fatty acids. Hence, these unsaturated fatty acids may be considered essential for the well-being of healthy children. The significantly lower blood levels for dienoic and tetraenoic acids in infants and children in fair and poor states of nutrition compared with healthy children not only indicate the inability of the children to synthesize these fatty acids but also indicate a dietary requirement for at least one of these essential fatty acids. As was pointed out previously from the results obtained with the control group of healthy children, there appears to be no dietary requirement for arachidonic acid when linoleic acid is supplied in the diet. Apparently human beings are able to convert linoleic acid to arachidonic acid in order to meet any metabolic requirement there may be for this fatty acid. Such a conversion has been postulated from feeding experiments with animals by a number of work-

ers (Nunn and Smedley-Maclean, '38; Rieckehoff, Holman and Burr, '49; Barki, Collins, Hart and Elvehjem, '49; Widmer and Holman, '50; Reiser, '51).

Since there appeared to be a general tendency for levels of dienoic and tetraenoic acids in the serum of healthy infants to be slightly lower than those for children over two years of age (Wiese, Gibbs and Hansen, '54), and since many of

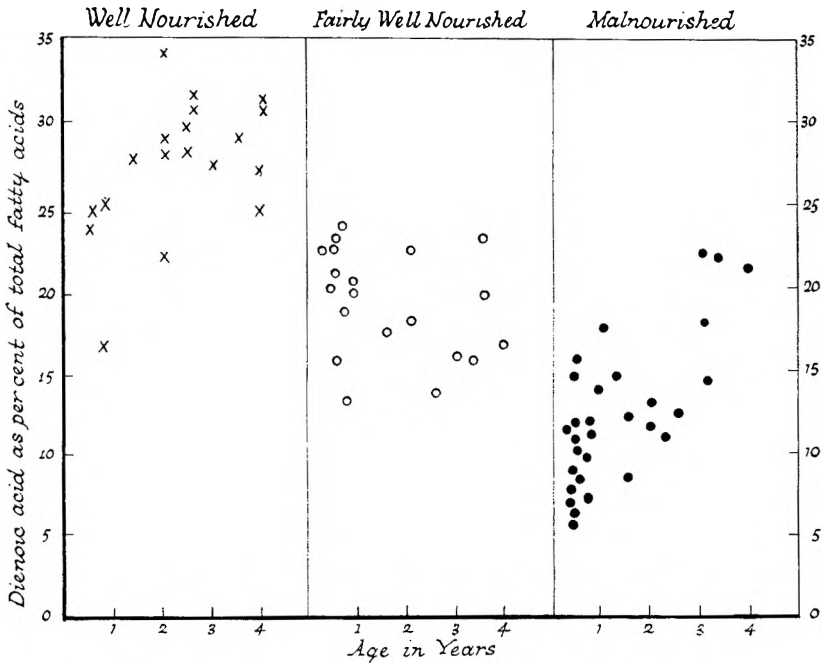

Fig. 1 Serum levels for dienoic acid in relation to age and nutritional state.

the children showing severe signs of malnutrition were young infants, scattergrams for the dienoic acid content of serum in relation to age were prepared for all children under 4 years of age. These are given in figure 1. Although the number of well-nourished infants studied is small, it will be noted that differences in the dienoic or linoleic acid content of the serum are very evident between the well-, fairly well- and poorly-nourished infants.

The significance of the higher level of trienoic acid in the serum of malnourished infants and children than in the serum of the control group is not apparent at present. However, again this finding parallels the results found for the serum fatty acids in fat-deficient and control dogs (Wiese and Hansen, '53).

The importance of the lower levels of hexaenoic acid in the serum of fairly well-nourished and malnourished children compared with those in the serum of healthy children is not known. Whether these fatty acids are derived from or are dependent upon the amount of two, three and/or 4 double-bond fatty acids cannot be stated. Conversion of trienoic to hexaenoic acid has been postulated for the rat by Widmer and Holman ('50) and for the chick by Reiser ('51). It is doubtful, however, that the presence of more hexaenoic acid in the blood serum of healthy children than in that of malnourished children indicates a dietary requirement for hexaenoic fatty acid.

Absorption data indicate the presence of small amounts of pentaenoic acid in the serum of malnourished children as well as in that of control children. The amount of this fatty acid could not be calculated because of the lack of a necessary $E^{1\%}_{1\,cm}$ standard. However, observed mean values for $E^{1\%}_{1\,cm}$ after subtraction of the contribution of hexaenoic acid at 3,475A are in the same range (three to 4) for all children, irrespective of nutritional status.

From the data presented for the serum fatty acids in well-, fairly well- and poorly-nourished infants and children, we believe the levels of dienoic, trienoic and tetraenoic acids, representing linoleic, linolenic and arachidonic acids, may serve as another chemical tool in the evaluation of nutritional status.

SUMMARY

The blood serum levels for the total fatty acids, dienoic, trienoic, tetraenoic and hexaenoic acids, were determined on 57 poorly-nourished infants and children.

There were no significant differences in the amount of the total fatty acids in serum of children in fair and poor nutritional states as compared with well-nourished children.

Dienoic, tetraenoic and hexaenoic acid levels in the serum of inadequately nourished children were significantly lower than in healthy children.

The trienoic acid level in the serum of inadequately nourished children was significantly higher than in healthy children.

Absorption data indicate the presence of a small amount of pentaenoic acid in the serum of all children. $E^{1\%}_{1\ cm}$ values at 3,475A due to pentaenoic acid alone did not vary with nutritional status.

ACKNOWLEDGMENTS

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AVAILABILITY OF AMINO ACIDS TO MICROORGANISMS

II. A RAPID MICROBIAL METHOD OF DETERMINING PROTEIN VALUE [1]

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TWO FIGURES

(Received for publication October 30, 1953)

It has been demonstrated (Horn, Blum, Womack and Gersdorff, '52) that different methods of processing affect the availability of the amino acids of cottonseed, whether measured by biological or microbiological methods. It was further shown that in order to get correlation between availability of essential amino acids to microorganisms and the results of animal feeding, it was necessary to use the sum of the availability of all the essential amino acids. This suggested that measurement of the change in protein value of a food resulting from processing or other treatment might be obtained by using an enzyme digest of the cottonseed meal to supply all the amino acids in the medium for growth of the microorganisms. If the same relative protein value could be obtained by this procedure as by previous methods, a rapid and inexpensive method would not only be available for determination of the nutritive value of cottonseed proteins but might also be adapted for other food proteins.

[1] Preliminary report given before the Animal Nutrition Research Council, October 14, 1953, in Washington, D. C.

In this paper it will be shown that microbiological growth curves provide a measure of the relative nutritional value of a protein and that this new method, which is both rapid and inexpensive, can be applied to the evaluation of the protein quality of cottonseed meal.

EXPERIMENTAL

Development of short method

The basal medium used contained only glucose, sodium acetate, salts A and B, adenine, guanine, uracil and 8 vitamins in the amounts given in a previous publication (Horn, Jones and Blum, '50). The microorganism used was *Leuconostoc mesenteroides* P-60. The preparation of the inoculum and assay procedure was that described in the above-noted publication (Horn et al., '50). The enzyme system described in previous publications (Agriculture Research, '53; Horn et al., '52) was used for digesting the meals. Preliminary assays showed that a concentration of approximately 700 mg of cottonseed protein per 500 ml gave satisfactory growth curves on microbiological assay. Since the maximum amount of the meal convenient to digest was 2 gm, all calculations were based on 2 gm of meal containing 6.35% nitrogen. The formula for the amount of meal to be used was $\frac{6.35 \times 2}{\%\ \text{N of meal}}$ = grams of meal used.

In converting nitrogen to protein in the cottonseed meal the factor 5.3 was used. Although the factor 6.25 is generally used in commerce, the factor 5.3 is more nearly correct, since the majority of the proteins in cottonseed contain over 18% nitrogen.

The experimental plan involved the use of the following digestions: (1) an enzymatic digestion of the processed meals; (2) an enzymatic digestion of an unprocessed meal used as a control standard; and (3) an acid digest of this same unprocessed meal as a second control standard. The enzyme digests were adjusted to pH 6.8 and made up to volume without filtering. The acid digest was filtered at pH 4.0 (Horn,

Blum, Gersdorff and Warren, '53), tryptophan added in the amount found in cottonseed, the pH adjusted to 6.8 and the solution made up to volume. Each digest was assayed at once in triplicate at each of 5 levels, giving a range of 1.35 to 6.75 mg of protein.

In the development of this method cottonseed meals number 1, 5, 6, 7, 8 and 9, and the standard of series 1 previously described (Horn et al., '52), were used. Series 2 [2] consisting of 9 meals was used to confirm the results obtained on series 1.

The rat feeding trials and relative protein efficiencies (grams of gain per gram protein consumed) were those described in a previous publication (Horn et al., '52).

RESULTS

The titration curves indicating microbiological growth are shown in figures 1 and 2 for meals in series 1 and 2, respectively. Using the data from these curves, calculations were made of the protein values of the processed meals as compared with the protein value of the unheated enzyme-digested meal.

Since complete digestion of the protein of the meal is not attained with the enzyme system used, the first step is to calculate the efficiency of digestion of the system. This is done by dividing the growth obtained (milliliters of 0.05 N alkali) from an enzyme-digested unheated meal by that obtained from a completely hydrolyzed meal (acid digest) at any convenient level. From figure 1, at the 5-mg level of total protein, the ratio of titration values for enzyme digest to acid digest (points A and B on curves) is $\frac{10.3}{14.0}$, or 0.73. Therefore, after enzymatic digestion, the amount of protein available to the microorganisms at the 5-mg level of total protein is $5 \times 0.73 = 3.65$ mg.

[2] Processed meals supplied as part of the research program inaugurated by the Southern Regional Research Laboratory in cooperation with the cottonseed industry.

The next step is to calculate the protein efficiency of each meal.

The term "protein efficiency," as used in this paper, denotes the microbiological growth (expressed in titration values) per milligram of *available* protein. To illustrate the method, all values have been calculated at the 5-mg level of total protein, which was shown above to be equivalent to 3.65 mg of available protein. Thus, at the 5-mg level of total

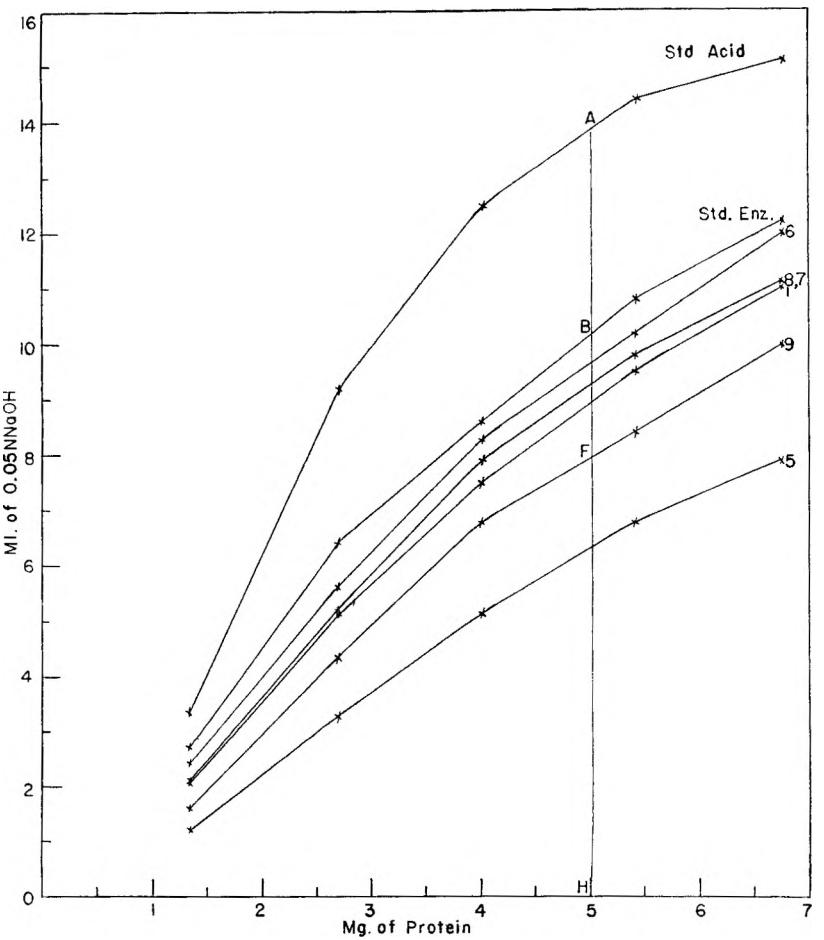

Fig. 1 Microbiological growth curves obtained from digests of series 1 cottonseed meals.

protein, the untreated meal has a protein efficiency of 2.82 (10.3 divided by 3.65). Meal no. 9 has an efficiency of 2.16 (7.9 divided by 3.65).

The indexes of protein values, then, are the ratios of the protein efficiency of each processed meal to the efficiency of the unprocessed standard meal. For example, for meal no. 9 the index is $\frac{2.16}{2.82} \times 100 = 76\%$.

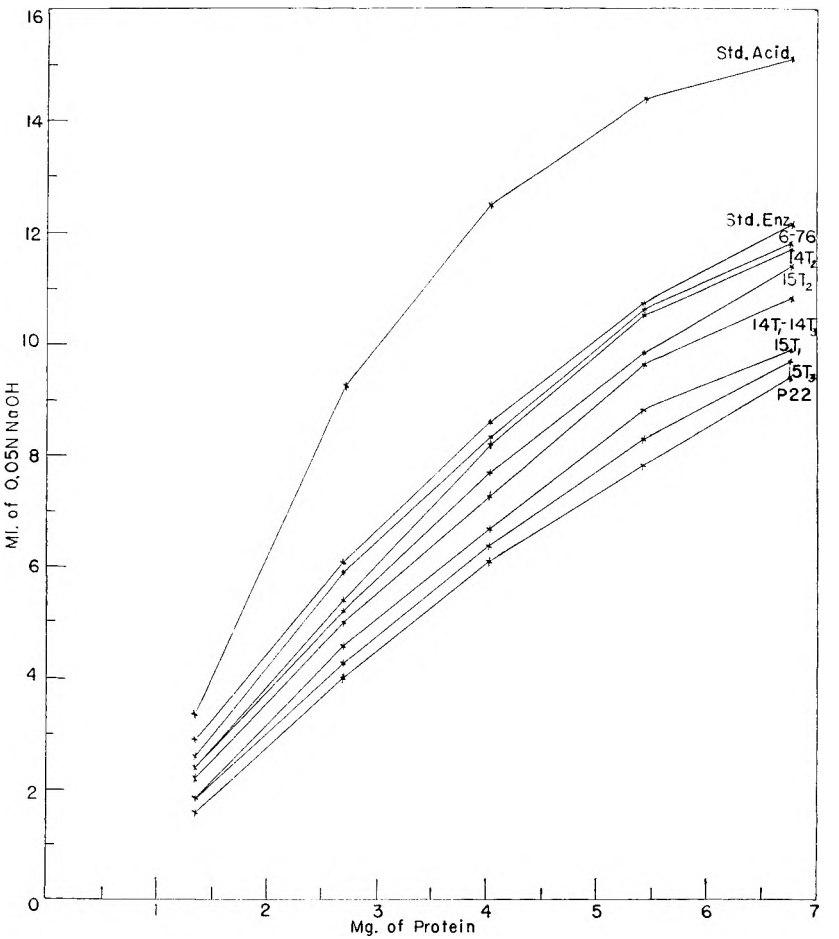

Fig. 2 Microbiological growth curves obtained from digests of series 2 cottonseed meals.

Table 1 compares the indexes of protein value of the processed meals in series 1 and 2, arranged in order of decreasing protein value, obtained by the microbiological and rat feeding methods, together with the conditions for processing each meal. It can be seen that with the exception of meal 15T1

TABLE 1

Processing data and results showing comparison of indexes of protein values from microbiological and rat growth studies

NUMBER OF COTTONSEED MEAL	N UNCORRECTED	PROCESSING CONDITIONS			INDEXES OF PROTEIN VALUE	
		Maximum temperature	Maximum cooking time	Amount of meal used	From microbiological studies	From rat growth studies
	%	°F.	min.	gm	%	%
		Series 1				
Standard [1]	10.0	..	..	1.27	100	100
6	6.34	200	20	2.00	93	90
8	6.66	180	70	1.91	90	86
7	6.47	200	70	1.96	90	80
1	6.75	234	20	1.88	86	73
9	6.68	240	76	1.90	76	68
5	6.35	279	100	2.00	61	22
		Series 2				
Standard		..	..	1.27	100	100
6-76 [2]	9.05	..	..	1.40	97	95
14T2	6.54	200	45	1.94	95	81
15T2	6.20	200	45	2.05	89	72
14T1	6.57	240	45	1.93	86	66
14T3	6.39	230	70	1.99	86	62
15T1	6.74	250	75	1.88	79	45
15T3	6.10	240	60	2.08	73	60
P22 [2]	7.85	230	60	1.62	70	59

[1] Gland-free meal, hexane-extracted.

[2] Butanone-extracted.

the meals are in the same order when arranged according to indexes of protein value obtained from microbiological and rat growth studies.

Unpublished experiments indicate that the method can be applied to all kinds of foods to detect changes in the nutritive value of protein due to heat, storage and processing.

SUMMARY

A rapid method for determining the nutritive value of the proteins of processed cottonseeds has been developed by making a microbiological assay, using an enzymatic digest of the meal as the only source of protein in the medium.

The method is based on the observation that the sum of the available amino acids determines the amount of growth of the microorganism. Since no amino acids are used in the basal medium, the method is relatively inexpensive and results can be obtained in a short time.

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A REFEREE BLOOD EXPERIMENT INVOLVING THE USE OF MICROCHEMICAL METHODS [1]

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INTRODUCTION

The Cooperative Nutritional Status Studies in the Northeast Region (Project NE_4, '47–'52) involved the use of physical, dietary and microchemical methods (Babcock et al., '52; Tucker et al., '52; Clayton et al., '53). In all of the bulletins reporting on the project the results were evaluated in the light of the precision of the original measurements and with consideration of the amount of uncontrolled or extraneous variation in the data. Attention was specifically drawn to the nature of the variable measured.

The microchemical methods used for blood analysis by the 6 state experiment stations taking part in the project were those of Bessey et al. (György, '50). Values were secured for hemoglobin, ascorbic acid, vitamin A and carotene from

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a variety of populations groups: grade school, high school and college students, pregnant women and industrial workers. Standardized techniques were suggested by the Subcommittee on Microchemical Methods for the project (Northeast Region, '51), since it was realized that slight changes are often introduced into chemical procedures by different technicians. These can cause noticeable differences in results on the same blood samples. Also, prior to the beginning of the project, facilities for training the laboratory workers were set up.

In June, 1950, after the blood tests for the cooperative project had been completed, the Technical Committee for the project voted to conduct a referee study in order to estimate the kind and amount of within-station and between-station variation in the microchemical determinations. By this time the laboratory staffs at some of the stations had changed and some of the new technicians had had little experience with the micromethods. However, it was felt that a referee study would be worthwhile, since it would help to determine the amount of variation in blood results which might be expected in a regional study.

The purpose of this paper is to present the findings, to describe the procedures used and the difficulties encountered and to make some suggestions for future studies of this type. A summary of the findings has also been published in the bulletin reporting the results of the blood tests for the cooperative project (Clayton et al., '53).

The referee study was planned and supervised by W. D. Foster, biometrician for the project. Preparation and distribution of the samples of blood serum and the statistical analysis of the results were carried out at the West Virginia Station.

The objectives of the study were as follows: (1) to estimate the consistency of techniques among stations, i.e., to learn if individual stations arrive at approximately the same figure for a given serum sample; (2) to estimate the consistency of technique within each station, i.e., to learn within

what limits the stations on an average can reproduce their own results; (3) as a secondary objective, to estimate the variation in readings on a single sample in a Beckman spectrophotometer.

The plan for the experiment provided for the identification of samples by number only. The schedule of replications and bloods and the intended statistical treatment were not released until the determinations had been reported.

PROCEDURE

The foremost criterion in procuring and distributing the serum samples was uniformity of procedure. This applied to obtaining the blood, centrifuging it, and distributing the serum into sample tubes, and to freezing, packing and mailing them. Thus, storage time, room temperatures and storage temperatures were kept as similar as possible for all the sets of samples in order to minimize the variation due to these effects. Also, each station was given the same set of instructions for procedure to follow upon receipt of the samples, so that the principle of uniformity could be extended as far as possible throughout the analysis.

Securing the blood

Forty to 50 ml of venous blood were drawn from each of three donors, centrifuged, and the serum divided into 4 replications for 8 sets of 12 samples each. Twelve milliliters of blood, secured from a 4th donor, provided serum for a 13th sample in each set. One set was sent to each of the 6 stations and two were kept in reserve for replacements.

Preparing the samples

With the strictest attention to the coded labels on the tubes, 0.5 ml of serum was pipetted into soft glass vitamin A tubes (approximately 10 cm long and with a 2.5-mm inside diameter) and the open ends closed with parafilm. The samples were stored at — 20°C. until they could be packed.

Packing and shipping

The 13 tubes of frozen serum for each station were taped together to form a compact unit and packed in dry ice in a gallon-size, insulated picnic jug. Large chunks of dry ice had been placed in the jugs to pre-chill them, after which the serum samples were placed in the middle of the jug, surrounded by the large chunks. Granulated dry ice was added to fill the interspaces. The jugs were then placed in their original cartons and smaller chunks of dry ice were packed into the corners, bottom and top of the cartons, so that dry ice was on both the inside and outside of the picnic jugs. The cartons in turn were packed into larger boxes insulated with approximately one and one-half inches of rock wool. All cartons were sealed with gum tape.

Such an elaborate packing procedure grew out of previous experimentation. It had been found that ordinary commercial picnic jugs, such as were used in this study, when filled with dry ice, would keep a sample frozen for only 24 to 28 hours. Even sealing the jug in its original carton with rock wool in the corners, bottom and top failed to keep the samples more than 34 to 36 hours. However, by using the procedure described above, the jugs repeatedly kept samples frozen as long as 80 hours.

Previous contact with the Morgantown Post Office had established the latest time for accepting packages in time for mailing and the packing procedure was scheduled accordingly. The Post Office advised that in this case shipment by rail, special handling, would be as fast as any other method. A dummy package sent to Orono, Maine, the most distant station, arrived in 42 hours.

In order to secure the blood and pack the samples in one day it was necessary to have 4 to 8 persons working constantly in order to keep on schedule.

Each station was instructed to unpack the samples as soon as they were received and to store them immediately at $-20°C$. Also each station was asked to send a telegram to

Dr. Foster giving the time of arrival and condition of the sample tubes.

All packages arrived within 48 hours. While some breakage of tubes during shipment was reported, the serum was salvaged in most cases. Replacement samples were sent to

TABLE 1

Results of the referee determinations

CATEGORY OF INTEREST	STATIONS						
	A	B	C	D	E	F	Av.
Ascorbic acid means, mg/100 ml							
Blood 1	0.21	0.28	0.14	0.16	0.23	0.20	0.20
Blood 2	1.07	0.89	0.78	0.74	0.88	0.78	0.86
Blood 3	1.15	0.92	0.76	0.80	0.92	0.77	0.89
Blood 4	0.66	0.75	0.48	0.65	0.65	0.54	0.64
Vitamin A means, μg/100 ml							
Blood 1	63	54	37	44	69	46	52
Blood 2	70	62	52	42	70	49	57
Blood 3	53	48	43	42	55	43	42
Blood 4	44	49	32	68	58	40	48
Carotene means, μg/100 ml							
Blood 1	124	126	109	129	140	105	122
Blood 2	230	217	196	234	239	187	217
Blood 3	234	234	198	231	244	204	224
Blood 4	96	100	88	111	116	89	109

Analyses of variance

SOURCE	DEGREES OF FREEDOM	ASCORBIC ACID MEAN SQUARE	VITAMIN A MEAN SQUARE	CAROTENE MEAN SQUARE
Stations	5	0.1165 ** [1]	1,134 **	3,622 **
Bloods	2	3.5803 **	602 *	77,776 **
S × B	10	0.0198 **	83 NS	151 NS
Within	52	0.0027	118	137
Ave. standard deviation		0.052	10.9	11.7
Coefficient of variation		8.0%	20.7%	6.2%

[1] * — significant at 5% level.
** — significant at 1% level.
NS — not significant.

two stations. Several improvements resulting from this experience are proposed under the section headed "Suggestions."

A summary of the results is given in table 1, which includes the means and analyses of variance for ascorbic acid, vitamin A and carotene.

RESULTS

Ascorbic acid

The analysis of variance showed that individually the stations obtained consistent results on the replications of each blood sample. However, there were obvious differences from station to station. A third kind of variation was the occasional departure of stations from maintaining relative positions from blood to blood. This effect can be seen by ranking the station values obtained for each blood and comparing the ranks. The coefficient of variation, which is the ratio of the standard deviation to the over-all mean, was 8.0%.

Vitamin A and carotene

The results for these two constituents are reported together because of the striking similarity in the patterns from station to station. For both, there was considerable variation among the determinations made at each station on replicates from the same blood. The variation from station to station was much larger. However, there was a strong tendency for each station to maintain the same relative position with respect to other stations from blood to blood. Stations that were high for one blood were consistently high for all bloods, and vice versa.

It is of interest to note that the coefficients of variation for vitamin A and carotene differed considerably. Actually, the standard deviation of a single determination on carotene was almost the same as that for vitamin A, but when each was compared to its respective mean, the carotene determinations appeared much more precise on a relative basis, slightly more so even than those of ascorbic acid.

Biological interpretation

The results of the present study cannot be interpreted biologically until they are compared with the results of experiments in which the variation in blood nutrient levels is examined over a period of time. Data offered by Dr. A. Hughes Bryan and Dr. B. C. Greenberg [9] of the University of North Carolina and by the Massachusetts Station [10] allow a comparison for blood serum ascorbic acid.

In the Bryan-Greenberg experiment, 4 adult subjects were studied on 4 different days over a period of three weeks while they were on a fairly uniform intake of ascorbic acid. Analysis of the results showed that the standard error of a single ascorbic acid determination on one individual on a single day as representative of the 4 days was 0.13 mg/100 ml.

In the Massachusetts experiment, 12 adult subjects were studied over a period of 10 days while they were on their normal diets. The average daily ascorbic acid intake of the individual subjects for the 10 days varied from 64 to 213 mg. There was also a wide variation in the daily ascorbic acid intakes of the individual subjects. Determinations of blood serum ascorbic acid were made on 5 different days and samples were taken at three different times on each day. The standard error of one determination on a single individual on one day as representative of 5 days was found to be 0.33 mg/100 ml (Eisenhart, '47).

The results for the referee study showed that the standard error of a single ascorbic acid determination on one blood for a single station, as representative of the 6 stations, was 0.24 mg/100 ml. Comparison of this standard error figure with those for the Bryan-Greenberg and Massachusetts experiments shows little difference among the three.

Data from the Massachusetts station also afforded a comparison for carotene.[11] The standard error of a single de-

[9] Special communication.

[10] W. D. Foster and Anne W. Wertz. Is one serum sample enough? Manuscript in preparation.

[11] See footnote 10.

termination on one person on one day as representative of several days was 16.3 μg/100 ml, while that for a determination on one blood for one station as representative of the 6 stations was 20.7 μg/100 ml. Again, the difference is not great.

The above findings indicate that for ascorbic acid and carotene the variation in results between stations was similar in magnitude to the variation in an individual from day to day.

TABLE 2

Comparison of the standard deviations of the chemical determinations with the concentrations of nutrients which correspond to the standard deviations of the Beckman readings

	ASCORBIC ACID	VITAMIN A	CAROTENE
	mg/100 ml	*μg/100 ml*	*μg/100 ml*
S.D. of chemical determinations	0.052	10.9	11.7
Equivalent of S.D. of Beckman reading	0.024	2.03	1.69

Rounding rule

Data from experiments like these, in which micromethods were used, also make possible the formulation of a rounding rule for the number of digits to be retained in reporting results. Using the statistical criterion of $\sigma/4$ (Statistical Research Group, '47), it was indicated that for hemoglobin [12] measured in units of grams per 100 ml, three digits including one decimal place are sufficient. Similarly, for ascorbic acid in units of milligrams per 100 ml, three digits including two decimal places are desirable. Both vitamin A and carotene in units of micrograms per 100 ml need at most three digits — units, tens and hundreds.

Variation in spectrophotometer readings

From the duplicate readings on the Beckman spectrophotometers, it was possible to estimate the average variation en-

[12] Data obtained from a previous experiment at the West Virginia, New York and New Jersey stations, 1950.

countered at the 6 stations. The standard deviations of the optical density readings were as follows: ascorbic acid 0.00185, vitamin A 0.00226 and carotene 0.00253. The concentrations of nutrients in serum corresponding to these standard deviations are compared with the standard deviations of the chemical determinations in table 2.

It will be seen that the Beckman readings tended to vary considerably less than the chemical determinations, of which the reading variation is a part.

Sources of station differences

In an attempt to determine the reasons for some of the station-to-station variation, a questionnaire was sent to the biochemist in charge of the laboratory at each station. The questions were concerned with the condition of the samples upon arrival and details of the procedures used in making the determinations.

By comparing the answers from the individual stations with the values reported in the referee study, it was possible to identify certain factors which probably accounted for most of the variation between stations. The following list, not necessarily in order of importance, is a summary of these factors:

1. Cracked or broken sample tubes, although serum was still frozen.
2. Incomplete mixing of serum after thawing. (Serum which has been frozen requires very thorough mixing to make it homogeneous.)
3. Variation in pipetting of serum and reagents.
4. Beckman differences due to battery trouble. (Two stations reported operating difficulty at the time of the study.)
5. Variation in ascorbic acid results due to differences in length of time between receipt of the serum samples and the measurement of the aliquots.
6. Failure to centrifuge all reagents in ascorbic acid determinations.

7. Variation in vitamin A and carotene due to differences in degree of extraction.

SUGGESTIONS FOR FUTURE REFEREE BLOOD STUDIES

The following suggestions, based on experience gained in this study, are offered for the guidance of others planning similar studies:

1. Secure containers with insulating qualities sufficient to keep the samples frozen as long as necessary without having to resort to the complicated and time-consuming process of packing with concentric boxes.
2. Protect the serum tubes from impact with dry ice. This could be done by packing them in a rigid container, such as a metal can, after wrapping them in sheet cotton and taping them together.
3. Use pyrex or hard glass tubes in shipping the serum samples instead of soft glass tubes.
4. Unpack tubes immediately on arrival and place in a freezer at — 20°C. or below.
5. Use a refrigerator to thaw serum samples to avoid breakage. (An ice bath was not satisfactory.)
6. Mix the serum thoroughly before measuring aliquots.
7. Take aliquots for all tests on the same day, adding trichloroacetic acid to the ascorbic acid aliquots and freezing until analyses are made. For repeat tests on ascorbic acid only frozen aliquots should be used, not left-over serum from the original vitamin A tube.
8. Insist that each participating laboratory run the analyses within three days after the samples are received.
9. Require that the temperature of each collaborator's freezer be — 20°C. or lower.

SUMMARY

As a part of the Cooperative Nutritional Status Studies in the Northeast Region (Project NE_4, '47–'52) a referee study was conducted to measure the variation in results of micro-

chemical analyses of blood serum for ascorbic acid, vitamin A and carotene.

Variations in replicate determinations within a station were found to be small compared with station-to-station variation. For each nutrient there was a tendency for the stations to follow a pattern, in which those reporting high values for one blood tended to report high values on the others and vice versa.

Evidence is given that the station differences were similar in magnitude to the day-to-day fluctuations in blood levels of ascorbic acid and carotene, as estimated from subsequently obtained data.

Probable sources of station-to-station variation are discussed and suggestions given for conducting future referee studies of this type.

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INTERRELATIONSHIP BETWEEN VITAMIN E AND PHOSPHORUS IN PREVENTING PEROSIS IN TURKEYS

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ONE FIGURE

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Scott ('53) reported that vitamin E, along with niacin, is required for the prevention of an enlarged hock disorder in turkeys fed diets of a practical or semipractical type. In later work by Hunt and Blaylock ('53), supplementation of a purified diet with vitamin E and niacin was not effective in preventing enlarged hocks in poults.

It was found by Slinger ('50) that phosphorus deficiency in chicks resulted in a widening of the tarsometatarsal ends of the tibiae and twisting of the tibiae and tarsometatarsi. While more properly described as a rachitic condition, the outward symptoms shown by these birds closely resembled the perosis which occurs in turkeys. Furthermore, both manganese and phosphorus additions to the diets aided in reducing the width at the tarsometatarsal ends of the tibiae and the amount of bone twisting.

That vitamin E influences phosphorus metabolism in the rat was indicated by the work of Weissberger and Harris ('43). These workers found that a deficiency or an overdosage of vitamin E in the diet brought about an increase in phosphorus turnover in bone and soft tissues. Furthermore, it has recently been reported by Schofield ('53) that phosphorus, along with vitamin E, is necessary for the prevention of muscular dystrophy in lambs and more particularly in calves.

On the basis of the above evidence it was thought possible that phosphorus may be necessary for the prevention of perosis in turkeys and that there may be an interrelationship between the mineral and vitamin E.

PROCEDURE

Day-old Broad Breasted Bronze poults were vent-sexed and weighed individually. They were then assigned to the various experimental groups so as to equalize sex and weight. Each group was comprised of 20 males and 20 females. The birds were reared in electrically heated battery brooders with raised wire floors for the first 4 weeks. At the end of this period they were moved to two brooder houses with similar pens supplied with wood shavings as litter and infra-red lamps as a source of heat. From 6 to 24 weeks of age the birds were allowed free access to these pens and to adjoining slatted sunporches.

Groups of birds were fed otherwise adequate diets containing all combinations of 0.6, 0.8 and 1.0% total phosphorus (calculated and confirmed by analysis) and 0, 2.5, 5.0 and 7.5 I.U. of supplementary vitamin E, as d-alpha-tocopheryl acetate, per pound. The levels of inorganic phosphorus were calculated to be 0.3, 0.5 and 0.7% in the starting and growing mashes, while the calcium content was kept constant at 1.6% for all mashes during both periods.

The composition of the basal mashes used from hatching through 8 weeks and from 8 through 24 weeks of age is shown in table 1. The desired level of calcium and the various levels of phosphorus were achieved by additions of ground limestone and defluorinated phosphate to these basal diets at the expense of a portion of the ground wheat. The major change in the starting mashes as compared with the growing mashes was a reduction in protein from about 28 to 20%. In addition, the starting mashes were fed through the first 8 weeks ad libitum but with no access to grain, while the

birds had free access to both the growing mashes and whole oats from 8 through 24 weeks of age. It should be emphasized that while the same levels of phosphorus and vitamin E were used in the starting and growing mashes, the concentrations in the total feed intake were considerably lower during the

TABLE 1

Composition of basal diets

INGREDIENTS	STARTING DIET, 1 DAY THROUGH 8 WEEKS	GROWING DIET, 8 THROUGH 24 WKS.
	%	%
Ground wheat	27.5	37.0
Ground yellow corn	15.0	10.0
Ground oat groats	5.0	...
Ground whole oats	..	10.0
Ground barley		10.0
Dehydrated cereal grass	3.0	6.0
Fish meal (65% protein)	2.0	1.0
Meat meal (50% protein)	2.0	2.0
Soybean oil meal (44% protein)	43.0	21.5
Dried buttermilk	2.0	2.0
Iodized salt	0.5	0.5
	gm/100 lb.	*gm/100 lb.*
Vitamin A oil (10,000 I.U./gm)	45.4	45.4
Dry vitamin D_3 (1500 I.C.U./gm)	45.4	45.4
Manganese sulfate (technical)	5.7	9.0
DL-Methionine (feed grade)	11.3	..
Riboflavin	0.15	0.15
Niacin	0.75	0.75
Vitamin B_{12} and penicillin supplement (3 mg B_{12} and 2 gm penicillin/lb.)	45.4	45.4
Sulfaquinoxaline	8.55	..

growing period due to dilution with oats. Insoluble grit and water were freely available at all times.

The birds were weighed individually every 4 weeks and feed consumption was recorded. The percentage incidence of perosis was noted on each occasion and a score of from one to 4 was assigned to each affected bird, depending on the severity. Birds with abnormalities such as bumble foot, pendulous crop or perosis, which were considered serious

enough to interfere with normal progress, were removed from the experiment at the time of weighing.

RESULTS

The weight and feed efficiency data at 8 and 24 weeks of age are presented in table 2. In general, raising the level of inorganic phosphorus from 0.3 to 0.5 or 0.7% resulted in

TABLE 2

Effect of vitamin E and phosphorus on growth of turkeys

TOTAL INORGANIC P	ADDED VIT. E	AVE. WT. (GM), 8 WEEKS			FEED/GAIN (0–8 wks.)	AVE. WT. (LB.), 24 WEEKS			FEED/GAIN (0–24 wks.)
		Males	Females	Unweighted mean		Males	Females	Unweighted mean	
%	*I.U./lb.*								
0.3	0	1,765	1,452	1,609 (39)[1]	2.29	22.33	14.30	18.3 (32)	4.14
0.3	2.5	1,806	1,407	1,607 (40)	2.32	22.19	14.32	18.3 (37)	3.96
0.3	5.0	1,807	1,395	1,601 (40)	2.23	21.34	13.42	17.4 (33)	4.04
0.3	7.5	1,766	1,442	1,604 (40)	2.33	20.61	13.93	17.3 (36)	4.13
0.5	0	1,941	1,596	1,769 (40)	2.19	21.89	14.88	18.4 (34)	3.93
0.5	2.5	1,977	1,550	1,764 (40)	2.20	23.12	14.27	18.7 (38)	4.02
0.5	5.0	2,023	1,621	1,822 (40)	2.14	22.68	14.66	18.7 (37)	4.04
0.5	7.5	2,050	1,556	1,803 (40)	2.12	22.21	14.19	18.2 (36)	4.01
0.7	0	1,998	1,533	1,766 (40)	2.18	22.08	14.17	18.1 (38)	4.02
0.7	2.5	2,108	1,607	1,858 (40)	2.09	23.01	14.46	18.7 (37)	3.89
0.7	5.0	2,082	1,543	1,813 (40)	2.13	22.75	14.26	18.5 (36)	4.19
0.7	7.5	1,997	1,552	1,775 (39)	2.20	21.98	14.27	18.1 (39)	4.11

[1] Parentheses show number of birds remaining.

an increase in the weight of the birds. Disregarding the concentration of vitamin E, and comparing the weights of the birds fed the lowest level of phosphorus with those of birds fed the higher levels by the "t" test (Snedecor, '46), indicated highly significant weight increases ($P < 0.01$) in males and females at both 8 and 24 weeks of age. Feed efficiency was also improved with the higher levels of phosphorus at 8 weeks but the difference was not apparent at 24 weeks of age. There were no appreciable differences in the weights or feed : gain ratios of birds receiving 0.5 and 0.7% inorganic phosphorus.

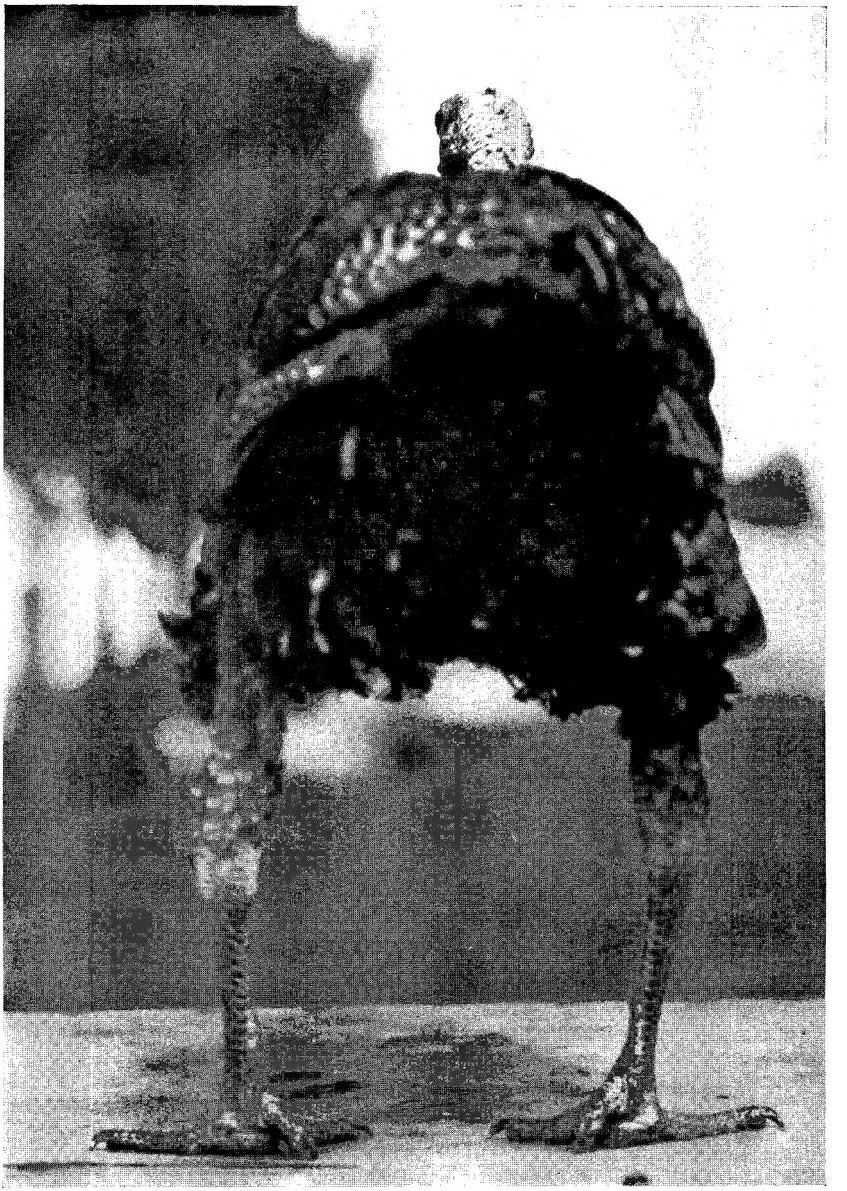

Fig. 1 A typical case of perosis, showing enlarged hocks and bowing of legs.

Disregarding phosphorus and comparing the weights of birds fed varying levels of vitamin E indicated no statistically significant differences at 8 weeks of age. At 24 weeks of age, the level of 2.5 I.U. of vitamin E per pound resulted in a weight significantly greater than that produced with no supplementary vitamin E ($P < 0.05$) in males but not in females. Other levels of vitamin E did not cause significant

TABLE 3

Effect of vitamin E and phosphorus on perosis in turkeys at 24 weeks of age

TOTAL INORGANIC P	PEROSIS SCORE	VITAMIN E ADDED (I.U. PER LB. OF MASH)				TOTALS
		0	2.5	5.0	7.5	
%						
0.3	Per cent [1]	52.6	30.0	35.0	20.0	34.2
	Index [2]	35.5	15.0	25.0	13.8	22.2
0.5	Per cent	26.3	20.0	25.0	11.3	21.5
	Index	17.1	17.5	10.0	13.8	14.6
0.7	Per cent	23.8	10.0	nil	nil	9.1
	Index	11.9	3.8	nil	nil	4.2
Totals	Per cent	33.9	20.0	21.1	11.9	
	Index	21.2	12.1	12.3	9.3	

[1] Percentage incidence based on number of males started.

[2] Severity index $= \frac{\text{Total score}}{\text{No. males} \times 4} \times 100$.

weight changes in either sex at 24 weeks of age. There appeared to be little or no interrelationship between phosphorus and vitamin E as far as growth was concerned.

The perosis observed in this experiment was outwardly characterized by an enlargement of the hock joints and a bowing of the legs, as is indicated in figure 1. In many cases the middle toe on each foot was curved inwards. The curved toe condition probably represents an attempt on the part of the bird to compensate for the difficulty in walking.

The effect of vitamin E and phosphorus on perosis at 24 weeks of age is shown in table 3. These data concern only males, since there was no incidence of perosis in the females.

Some transitory perosis was evident at 4 weeks of age in the males fed the lowest level of phosphorus but this condition had largely disappeared by 8 weeks of age. Permanent cases began to appear between 12 and 16 weeks of age but the most critical period for the development of the condition was between the ages of 16 and 20 weeks. The results indicate that vitamin E was of some value in preventing perosis at all levels of phosphorus. However, complete prevention was achieved by the vitamin only with the diet containing 0.7% inorganic phosphorus. Similarly, while raising the level of phosphorus was in itself beneficial, complete protection required the presence of supplementary vitamin E.

DISCUSSION

The results of this work confirm the observation of Scott ('53) that vitamin E is necessary for the prevention of perosis in turkeys. It is also apparent that phosphorus plays an important role in this connection. It is of interest that while 0.5% inorganic phosphorus was adequate for maximum growth, this level was too low for the prevention of perosis. It may well be that phosphorus is the primary factor involved in perosis prevention and that vitamin E functions in the regulation of phosphorus metabolism.

The National Research Council ('50) recommends an allowance of 1.0% phosphorus for starting turkeys, with the stipulation that 0.4% of the total should be inorganic in nature. Calculation of the amount of inorganic phosphorus present in the starting and growing diets used by Scott ('53) gave values of 0.84 and 0.64%, respectively. Furthermore, the growing diet was fed without access to grain. The use of diets adequate in available phosphorus possibly accounts for the effectiveness of vitamin E in that work.

The failure of vitamin E and niacin to prevent the condition in the experiments of Hunt and Blaylock ('53) cannot be explained on the basis of lack of phosphorus, since their diets contained in excess of 1.14% inorganic phosphorus. They were able to produce enlarged hocks by supplying the

protein in the purified diet in the form of isolated soybean protein. If the protein source was replaced by another isolated soybean protein or soybean oil meal, the incidence of enlarged hocks was reduced to zero. The isolated soybean protein that did not produce the enlarged hocks was washed with 16 volumes of water before being incorporated into the diet; therefore, it would have a greatly reduced inorganic phosphorus content. These results seem difficult to explain on the basis of phosphorus level. However, it has been shown (Caskey and Norris, '38) that too much calcium and phosphorus causes perosis in chicks. It may be that the diets of Hunt and Blaylock ('53) were sufficiently high in calcium and phosphorus to cause enlarged hocks and that prevention of the disorder was brought about by lowering the levels of these minerals. On the other hand, factors other than vitamin E, niacin or phosphorus may have been involved in the enlarged hock disorder observed by these workers.

The present results do not necessarily mean that the inorganic phosphorus content of mash used through the growing period need be as high as 0.7%. It is entirely possible that if the birds receive sufficient phosphorus in the starting period, a lower level would suffice later on. More work will be necessary to clarify this point.

SUMMARY

Groups of turkeys were fed mashes containing all combinations of 0.3, 0.5 and 0.7% inorganic phosphorus and 0, 2.5, 5.0 and 7.5 I.U. of vitamin E per pound from hatching through 24 weeks of age. Grain was fed with the mash for the 8- through 24-week period.

Based upon the growth data, there appeared to be little or no interrelationship between vitamin E and phosphorus. Levels of 0.5% inorganic phosphorus and 2.5 I.U. of vitamin E per pound of feed appeared adequate for optimum growth but were not sufficient for perosis prevention.

Both vitamin E and phosphorus aided in reducing the incidence and severity of perosis, with complete prevention

being achieved when the diets contained 0.7% inorganic phosphorus and 5.0 I.U. of supplementary vitamin E per pound of feed.

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THE RIBOFLAVIN REQUIREMENT OF THE BABY PIG [1]

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ONE FIGURE

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Hughes ('40), Krider et al. ('49), Mitchell et al. ('50) and Miller and Ellis ('51) have reported riboflavin requirements for growing pigs beyond weaning age. Lehrer and Wiese ('52), studying riboflavin deficiency in baby pigs, suggested the riboflavin requirement as being near 3.0 mg daily per 100 pounds of body weight. Only 8 pigs were used in this study. Forbes and Haines ('52) have concluded that the riboflavin requirement of the baby pig at 85°F. and 70% relative humidity lies between 1.5 and 2.0 μg per gram of feed dry matter. A modified paired-feeding method for equalizing intake was utilized.

Since the only extensive data presented to date relative to determination of the riboflavin requirement of the baby pig have been obtained using a limited feeding procedure, it seemed wise to consider the requirement under more liberal feeding conditions. The purpoes of the present paper is to present such data.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article 1574.

[2] The data contained in this paper comprise a portion of the research and thesis to be presented by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy, School of Graduate Studies, Michigan State College, East Lansing.

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EXPERIMENTAL

Three experiments involving 52 pigs were conducted. Only the third of these three experiments will be described here. The first two experiments were similar to the third except that they were designed to establish trends in the riboflavin requirement of the baby pig and to develop techniques and establish standards for reducing experimental error.

Seven Chester White-Duroc Jersey crossbred pigs and 12 Chester White-Yorkshire crossbred pigs from two litters were used in the experiment. All pigs were sired by the same Chester White boar and were taken from the sow when 72 hours old and placed in individual cages. Room temperature was thermostatically maintained at 70°F. and infra-red heat lamps were used to supply additional heat for the first two weeks of the experiment. No attempt was made to maintain a constant percentage of relative humidity.

All pigs first underwent a 4-day depletion-adjustment period during which they were fed a synthetic milk diet described in table 1. The purpose of the depletion-adjustment period was partially to deplete the riboflavin reserve of the pig and also to allow for adjustment by the pigs to the cages, to the milk and to the feeding method. At the end of the depletion-adjustment period, the pigs were placed into individual cages and assigned to lots on the bases of sex, size, litter, feed intake, weight gained during the depletion-adjustment period and general appearance. At this time the pigs were 7 days old.

It was quite clear from the two preliminary experiments as well as from reports of other workers under a variety of conditions that the minimum riboflavin requirement of the baby pig for normal growth was not in excess of 4 mg of riboflavin per kilogram of solids consumed. Consequently, this level was chosen as the positive control level in this experiment. Three pigs were placed on the basal diet containing no riboflavin and 4 pigs were placed on each of 4 diets containing the following levels of riboflavin, in milligrams per kilogram of solids: 1.0, 2.0, 3.0 and 4.0, with the animals fed the 4.0-mg level being designated as the positive

control group. All pigs were fed regularly 6 times a day an amount of diet which they would consume in the 4-hour period. The amount of diet fed was limited only when scouring appeared. Scouring was not a particular problem after the first week of experimental feeding. All feed was carefully

TABLE 1

Composition of basal synthetic milk diet (15.1% solids in water)

CONSTITUENTS	AMOUNT
Casein, Labco (%, dry basis)	30
Lard (%, dry basis)	10
Mineral mixture [1] (%, dry basis)	6
Cerelose (%, dry basis)	54
Water-soluble vitamins (mg/kg of "milk"):	
Thiamine	0.65
Niacin	2.50
Pyridoxine	0.65
Calcium pantothenate	3.00
Para-aminobenzoic acid	2.60
Inositol	26.00
Choline	260.00
Biotin	0.01
Pteroylglutamic acid	0.052
Ascorbic acid	16.00
Fat-soluble vitamins (am't./kg of "milk"):	
Vitamin A	2,000 I.U.
Vitamin D	200 I.U.
α-Tocopherol acetate	1.00 mg
2-Methyl-1, 4-naphthoquinone	0.28 mg

[1] Mineral mixture used by Johnson et al. ('48), except that Ca lactate replaced $CaCO_3$ in equimolar amount.

measured. Samples of each diet were microbiologically assayed for riboflavin at frequent intervals to verify the prepared concentration. Determinations were made using the method of Snell and Strong ('39) and samples were prepared according to the directions of Strong and Carpenter ('42).

Pigs were weighed every 4th day before the final feeding. Blood samples were taken from an ear vein during the first and final weeks of the experiment for cell counts and hemoglobin determination.

RESULTS AND DISCUSSION

The most important results of the experiment are presented in table 2. Statistical treatment was given, using the method of Snedecor ('46) for analyzing single classification variance.

TABLE 2

Response of baby pigs to synthetic milk diets containing different levels of riboflavin [1]

	LEVEL OF RIBOFLAVIN IN DIET, IN MG/KG SOLIDS				
	0	1	2	3	4
Lot number	1	2	3	4	5
Number of pigs	3	4	4	4	4
Days on test	28	28	28	28	28
Ave. initial wt.[3] (lb.)	6.46±0.75 [2]	6.30±0.75	6.28±0.27	6.27±0.44	6.07±0.39
Ave. final wt.[4] (lb.)	6.86±0.69	10.24±1.20	14.31±0.68	18.21±0.70	17.31±0.79
Ave. daily gain [5] (lb.)	0.01±0.004	0.14±0.03	0.29±0.03	0.43±0.01	0.40±0.03
Ave daily feed consumed (lb.)	0.22±0.005	0.33±0.02	0.45±0.02	0.57±0.01	0.53±0.02
Solids per lb. gain [6] (lb.)	17.91±3.93	2.48±0.31	1.59±0.12	1.33±0.03	1.32±0.04

[1] Pigs were taken from sow when 72 hours old and placed on depletion diet containing no riboflavin for 4 days. Pigs were then assigned to lots and started on feeding trial.

[2] Standard error of mean.

[3] Weight at 7 days of age.

[4] Weight at 35 days of age.

[5] There was a significant difference in daily gain at the 1% level between all lots except lots 4 and 5.

[6] All lots were significantly more efficient in food utilization at the 1% level than the lot receiving no riboflavin. Lot 3 was significantly more efficient at the 5% level than lot 2. Lots 4 and 5 were significantly more efficient at the 1% level than lot 2.

All of the pigs on the basal diet containing no riboflavin and all of the pigs receiving 1 mg of riboflavin per kilogram of solids showed gross external symptoms of riboflavin deficiency. These pigs exhibited a rough haircoat, a heavy sebaceous exudate about the eye and ear and a dried, somewhat caked exudate on the skin (fig. 1). These pigs became

Fig. 1 The pig on top received no riboflavin in the diet, while the animal below received 3 mg of the vitamin per kilogram of solids.

weak and thin and had a higher incidence of diarrhea than the pigs on the other diets. The dietary intake of the pigs receiving no riboflavin gradually decreased, until at the end of the experiment they were consuming less than 75 gm of solids per pig per day, or an amount approximating only 2% of the animal's body weight. Thus, the pig in this state of acute anorexia is undoubtedly influenced by a multiple nutrient deficiency.

Evidence of the existence of a multiple nutrient deficiency was provided by the treatment and subsequent response of one of the pigs in lot 2 of this experiment. This pig, after 21 days of experimental feeding, had a greatly reduced appetite, was very thin and weak and lay in a comatose state near death. An intraperitoneal injection of 10 ml of a riboflavin-free B vitamin preparation was given, containing (in milligrams per milliliter): nicotinamide 5; calcium pantothenate 5; thiamine 2; pyridoxine 1; folic acid 0.25; *p*-aminobenzoic acid 0.25; inositol 1; and vitamin B_{12} 0.005. Within 12 hours the pig was able to walk, became much more alert and showed an improved appetite. When a similar intraperitoneal injection was given three days later, the pig continued to respond to treatment. However, during the 4-week experimental period this animal had gained less than three pounds.

In order to determine whether the deficiency symptoms exhibited by this pig could be alleviated, the animal was fed the diet of the positive control lot containing 4 mg of riboflavin per kilogram of solids. In addition, a 10-ml injection of the previously described multiple vitamin preparation was given on the first and second days, followed by an intraperitoneal injection of 100 mg of riboflavin every 4th day of the 19-day recovery period. The animal's appetite improved rapidly and during the recovery period he gained 0.5 lb. per day, which approaches the normal growth curve presented by Ittner and Hughes ('38). Similar results were obtained by this procedure with two other pigs from lot 2 and two pigs from the basal lot.

One pig from the basal lot and one pig from lot 2 were sacrificed at the end of the 4-week experimental feeding period. In each instance, the liver and kidneys were somewhat mottled and showed evidence of fatty infiltration. In addition, there was excessive fluid in the peritoneal cavity and the pericardial sac. The small intestine, cecum and colon were congested and edema was present in the spiral colon.

The tissues taken at post mortem were placed in formalin, embedded in paraffin and stained with hematoxylin-eosin. Nerve tissue was also stained, using Weil's ('28) method.

Lesions of the eye were confined to the lens, cornea and eyelids. Cataracts were present in the lens at 35 days of age. The cataracts were located posterior to the equator in the zone where the posterior surface of the lens begins to be devoid of epithelium. They were manifested by a swelling and separation of the lens fibers. The epithelial cells, possessing nuclei, were enlarged, elongated and formed the so-called vesicular cells found in cataractous conditions. Ballooning of the columnar cells of the basal layer of the corneal epithelium was observed. There was no evidence of vascularization of the cornea or depigmentation of the iris. There was a ballooning of the tubular glands of the eyelids.

Myelin sheath degeneration was not observed in sections from the cerebrum, cerebellum, thalmus, mid-brain, spinal cord, dorsal root ganglia and sciatic nerves.

The skin showed varying degrees of atrophy and hyperkeratosis of the stratum mucosum. Mucinous degeneration was present in the cecum and colon. Hemorrhage was very prominent in the rectum. Studies of the white cell counts and the hemoglobin determination values revealed no significant trend that could be related to the level of riboflavin in the diet.

Pigs receiving 2.0, 3.0 and 4.0 mg of riboflavin per kilogram of solids in the diet showed no gross symptoms of riboflavin deficiency. However, those pigs receiving 2.0 mg of riboflavin per kilogram of solids gained significantly ($P =$

0.01) less rapidly than did the pigs receiving 3.0 and 4.0 mg per kilogram of solids. Furthermore, their efficiency of feed utilization was 20% less than that of pigs in the two lots receiving higher levels of riboflavin. There were only slight differences between pigs fed 3.0 mg of riboflavin per kilogram of solids and pigs in the positive control group. In all but one instance, these slight differences were in favor of the former group. The one exception was in efficiency of feed utilization. It is apparent that under the conditions of this experiment the riboflavin requirement of the baby pig for normal growth approximates 3.0 mg per kilogram of diet. This is a definitely higher requirement than that of 1.5 to 2.0 mg per kilogram of diet reported by Forbes and Haines ('52).

The higher riboflavin requirement found in this study as compared with that reported by Forbes and Haines ('52) may be due to a number of factors. The environmental temperature in this experiment was 5° to 15°F. lower than that reported by the above authors. Mitchell et al. ('50) have shown that the riboflavin requirement of growing pigs is higher at lower temperatures. Moreover, uncontrolled relative humidity may have been a factor accounting in part for our higher requirement. In addition to the above factors, variation in the genetic make-up of the animals used and the more liberal feeding method employed may have influenced the vitamin requirement found in this study.

SUMMARY

Nineteen baby pigs were used in an experiment to determine the riboflavin requirement. Following a depletion-adjustment period on a riboflavin-free, synthetic milk diet, the pigs were individually fed diets containing 0, 1, 2, 3 and 4 mg of riboflavin per kilogram of solids. The feeding method was essentially ad libitum.

Data on individual growth response and dietary intake were recorded. Analysis of the data indicates that the riboflavin requirement of the baby pig for optimum growth and

feed efficiency approximates 3.0 mg per kilogram of solids. External, gross and microscopic lesions were present only in those animals receiving less than 2.0 mg of riboflavin per kilogram of solids. Deficiency symptoms could be alleviated by riboflavin supplementation.

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DIET AND THE METABOLISM OF 2-AMINOFLUORENE [1]

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The carcinogen, 2-acetylaminofluorene, inhibits the uptake of S^{35} DL-methionine by rat liver slices, an inhibition which is overcome by riboflavin (Wase, Allison and Migliarese, '52). Feeding this amine to animals produces a riboflavin deficiency and reduction in protein stores (Griffin et al., '49; Allison et al., '50), toxic effects which can be overcome in whole or in part by increasing dietary riboflavin and protein (Wase and Allison, '50; Wase, '52). A dietary deficiency in riboflavin or in pantothenate reduces the excretion of urinary conjugated amines in animals fed 2-aminofluorene or the acetylated derivative, demonstrating an effect of diet upon the metabolism of these compounds (Allison and Wase, '52). The following experiments were planned to determine in more detail the effect of dietary protein, riboflavin and pantothenate on the end products of the metabolism of 2-aminofluorene.

METHODS

Table 1 is a record of the basal diets fed to the dogs. Diet A contained an amount of casein that would maintain nitrogen equilibrium in an average dog, whereas diet B had a quantity of casein that would promote growth in puppies and regeneration of protein stores in an adult. Both diets contained the vitamin content found to be adequate for normal growth of puppies.

[1] Supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

The technique for making the diet follows: The diet pan and spoon were weighed together. About two-thirds of the total water was added to the pan, together with the agar, the mixture being heated until the agar dissolved. The lard was weighed, cut into chunks and added to the agar solution. The heat under the pan was removed and while the lard melted, the carbohydrate, protein and salt mixture were mixed together. This mixture was added to the agar solution containing the dispersed lard. The pan with the diet mixture and

TABLE 1

Basal diet fed to dogs

INGREDIENTS	AMOUNT		VITAMINS	AMOUNT
	A	B		
	gm	*gm*		*mg/1000 gm of dry diet*
Casein (vitamin-free)	63	250	Thiamine	2.0
Sucrose	166	...	Riboflavin	1.6
Dextrose	387	366	Nicotinic acid	16.0
Dextrin	187	187	Calcium-pantothenate	13.0
Lard	153	153	Pyridoxine	1.0
Salt mixture [1]	17	17	Choline	1000.0
Agar	27	27	2-Methyl-naphthoquinone	0.0006
Distilled water	1,400	1,400	Alpha-tocopherol	30.0
			Biotin	0.6
			Folic acid	0.6
			Vitamin A	55,000 units
			Vitamin D	11,000 units

[1] Wesson ('32).

spoon were weighed and water added according to the amount recorded in table 1. The liquid diet was mixed with an electric stirrer until it thickened, the vitamins were added and the mixture was poured into pans and placed in a refrigerator.

The protein was replaced by an equivalent number of sucrose calories in diet A to make the protein-free diet. The effect of varying amounts of protein in the diet was studied by feeding 8 dogs high protein diet B, 9 dogs maintenance diet A and 5 dogs the protein-free diet for a period of approximately 6 weeks. The effects of varying amounts of ribo-

flavin were determined by feeding 7 dogs riboflavin-free diet A for 8 weeks, followed by 6 weeks on diet A with the riboflavin intake increased to 2.5 mg/kg/day. Also 5 other dogs were fed diet B with the riboflavin intake increased to 2.5 mg/kg/day for 4 weeks. Variation in pantothenate was studied by feeding 5 dogs pantothenate-free diet A for 8 weeks, followed by an increased pantothenate intake of 20 mg/kg/day for 6 weeks.

Each dog received orally a gelatine capsule containing 2-acetylaminofluorene (30 mg/kg body weight) two weeks before being removed from the diet under study, and similarly, one week later, a capsule of the same quantity of 2-aminofluorene.

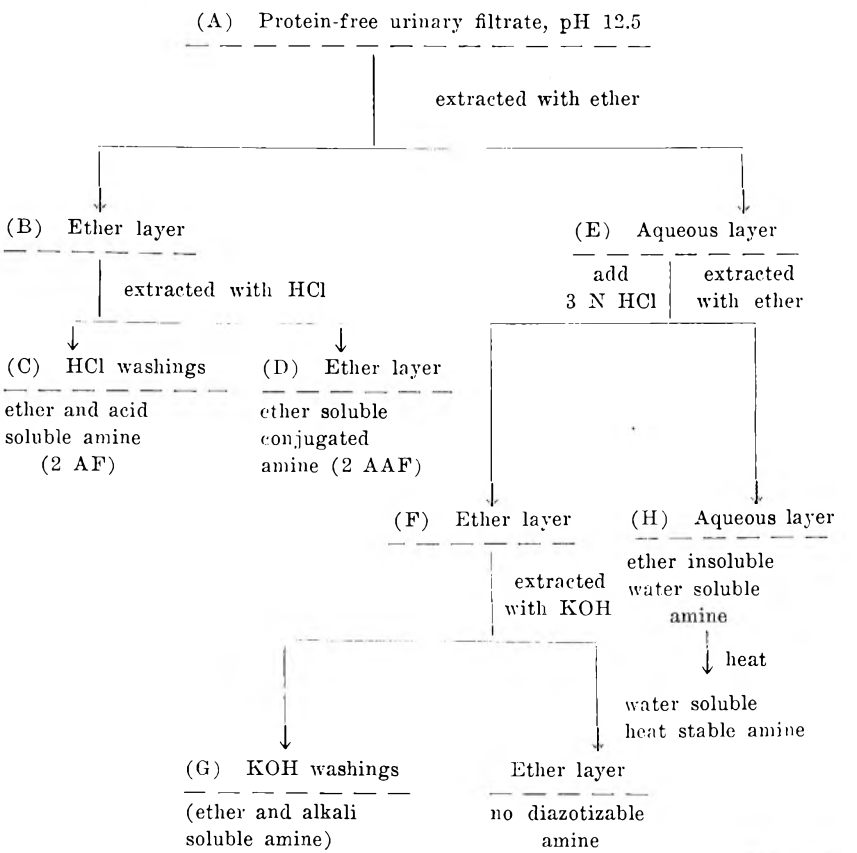
Following the ingestion of the carcinogen, urine was collected over a period of three days. The urine samples were treated with 20% trichloracetic acid (one part of acid solution to 9 parts urine), diluted to 2 l and filtered through paper. Three milliliters of 40% NaOH were mixed with an aliquot (150 ml) of urine filtrate, giving a protein-free urine filtrate of pH 12.5 (A; see table 2). One hundred milliliters of this filtrate were extracted with ether until all ether-soluble amines had been removed. A free diazotizable amine could be extracted from the ether solution (B) with normal HCl, a free amine that had the same absorption spectrum in the ultraviolet as 2-aminofluorene, with a major peak at 260 and minor peaks at 290 and 300 mμ. The acid washings (C) were brought to 100 ml with N HCl and the amount of free amine determined as 2-aminofluorene through ultraviolet absorption. The spectrum for the ether-soluble, acid-insoluble fraction (D) was identical with that for 2-acetylaminofluorene, with a peak at 275 mμ. Thus the acid-extracted ether solution (D) was diluted to 250 ml and the amount of conjugated amine determined quantitatively as 2-acetylaminofluorene through ultraviolet absorption. The ether fraction (D) also could be hydrolyzed with HCl, forming the free amine, which

then could be diazotized with Naphthanil ASD [2] and determined colorimetrically according to the technique described by Allison and Wase ('52). Using these methods, 2-acetylaminofluorene and 2-aminofluorene could be recovered 98 to 99% when added to control urine.

The ether-extracted, alkaline urine solution (E) had a diazotizable amino derivative or derivatives which could be extracted with ether from acid solutions; hence the aqueous

TABLE 2

Urinary fractionation



[2] Ortho-toluidide of 2-hydroxy, 3-naphthoic acid, available from E. I. DuPont de Nemours Co.

layer was made acid by adding 3 ml of concentrated HCl. A diazotizable amine was extracted into ether (F), an amino derivative which could be put back into alkaline solution with 1 N KOH, a solubility which suggests the presence of acidic groups. The alkaline washings (G) were diluted to 50 ml and the diazotizable amine determined with Naphthanil ASD. The color formed seemed to be a function of the presence of the Naphthanil group and independent of the type of amino derivative obtained from the urine, so that the amines were determined by using 2-aminofluorene as a standard.

The ether-extracted acid urine solution (H) still had diazotizable amines in solution, amines which were ether-insoluble and quite water-soluble. One or more of these derivatives could be extracted with butanol to give a peak at 285 mμ in the ultraviolet. The butanol-soluble compound was heat-labile, being destroyed by heating in an acid solution. Thus an aliquot (2 ml) of acid urine (ether-extracted) was diazotized to determine the free amine derivatives. Another portion (2 ml) was hydrolyzed for one hour in a boiling water bath following the addition of 1 ml of 3 N HCl. The heated urine was diazotized and the remaining free amine calculated from a 2-aminofluorene standard curve as water-soluble, heat-stable amine. The loss in diazotizable amine due to heating in an acid solution was calculated as water-soluble, heat-labile amine, a loss which was exactly equivalent to the amount dissolved in butanol.

RESULTS

The effects of the casein content of the diet upon urinary metabolites of 2-acetylaminofluorene and 2-aminofluorene are illustrated in table 3. These data demonstrate that the excretion of ether-soluble, acid-soluble amine, which was identified spectrophotometrically as 2-aminofluorene, increased as the protein content of the diet increased. Similarly, the ether-soluble conjugated amine, identified spectrophotometrically as 2-acetylaminofluorene, increased with dietary protein intake. Relatively little change was noted in the excretion of

TABLE 3

Urinary metabolites of 2-acetylaminofluorene (2 AAF) and 2-aminofluorene (2 AF) when administered to dogs fed various amounts of protein and riboflavin [1]

RIBOFLAVIN INTAKE	DIETARY PROTEIN	NO. DOGS	ETHER- AND ACID-SOLUBLE AMINE	ETHER-SOLUBLE CONJUGATED AMINE	ETHER- AND ALKALI-SOLUBLE AMINE	WATER-SOLUBLE, HEAT-STABLE AMINE	WATER-SOLUBLE, HEAT-LABILE AMINE
mg/kg/day	*%*		*mg/kg body weight*				
			Urinary metabolites of 2 AAF				
0.025	0	5	0.18 ± 0.02 [2]	0.10 ± 0.03	0.27 ± 0.02	0.38 ± 0.03	0.18 ± 0.04
0.025	6.3	9	0.27 ± 0.02	0.25 ± 0.05	0.21 ± 0.02	0.39 ± 0.04	0.06 ± 0.01
0.025	25	8	0.34 ± 0.04	0.24 ± 0.05	0.25 ± 0.03	0.56 ± 0.05	0
0	6.3	7	0.17 ± 0.02	0.06 ± 0.005	0.28 ± 0.03	0.39 ± 0.02	0.52 ± 0.11
2.5	6.3	7	0.34 ± 0.03	0.24 ± 0.05	0.26 ± 0.02	0.64 ± 0.03	0
2.5	25	5	0.34 ± 0.02	0.25 ± 0.02	0.29 ± 0.1	0.67 ± 0.04	0
			Urinary metabolites of 2 AF				
0.025	0	5	0.27 ± 0.01	0.05 ± 0.01	0.30 ± 0.01	0.82 ± 0.02	2.39 ± 0.09
0.025	6.3	9	0.33 ± 0.04	0.05 ± 0.01	0.26 ± 0.02	0.51 ± 0.04	1.34 ± 0.09
0.025	25	8	0.90 ± 0.17	0.12 ± 0.01	0.32 ± 0.02	0.58 ± 0.06	0.94 ± 0.19
0	6.3	7	0.26 ± 0.01	0.05 ± 0.01	0.46 ± 0.01	0.72 ± 0.03	2.20 ± 0.19
2.5	6.3	7	0.56 ± 0.08	0.12 ± 0.01	0.34 ± 0.02	0.57 ± 0.01	0.38 ± 0.02
2.5	25	5	0.95 ± 0.27	0.11 ± 0.01	0.31 ± 0.02	0.65 ± 0.02	0

[1] Dosage of carcinogens = 30 mg/kg body weight.

[2] Standard error.

ether- and alkali-soluble amine. The excretion of water-soluble, heat-labile amines, on the other hand, decreased as the amount of dietary protein was raised. The excretion of these heat-labile amines was most marked when the free amine was fed. Possibly the greater toxicity of the free amine (Allison and Wase, '52) may be associated with the formation of this heat-labile derivative. The possible advantage of a high protein diet, over and above that needed to maintain nitrogen equilibrium in an average dog, is demonstrated by these data.

The effects of varying amounts of dietary riboflavin on the excretion of diazotizable amines are illustrated in table 3, data which demonstrate the greater excretion of the free and conjugated amine associated with increased riboflavin. Reduced dietary riboflavin together with reduced protein intake favored the excretion of the water-soluble, heat-labile fraction, a result which suggests that the protective effects of dietary riboflavin and protein may be associated with these alterations in the metabolism of the carcinogen. The reduced excretion of water-soluble, heat-labile amine may be correlated with more complete oxidation and thereby detoxication of these compounds. It was suggested by Shils et al. ('50) that raising the riboflavin content of the diet may increase the over-all enzyme activity of the liver. The data in table 3 emphasize again the possible advantage of sometimes increasing a dietary constituent above the amount ordinarily considered adequate.

Riggs and Hegsted ('48, '49) and Shils et al. ('49) demonstrated a marked effect of dietary pantothenate upon the conjugation of aromatic amines in the rat. Allison and Wase ('52) reported an increased excretion of conjugated 2-aminofluorene associated with high pantothenate intake in dogs. The data in table 4 support these reports. These data also suggest the possibility that the increased excretion of water-soluble, heat-labile amine is a function of the degree of conjugation; the smaller the amount of urinary conjugated amine the larger the quantity of the heat-labile derivative.

TABLE 4

Urinary metabolites of 2-acetylaminofluorene (2 AAF) and 2-aminofluorene (2 AF) when administered to dogs fed various amounts of pantothenate and 6.3% casein in the diet [1]

PANTOTHENATE INTAKE	NO. DOGS	ETHER- AND ACID-SOLUBLE AMINE	ETHER-SOLUBLE CONJUGATED AMINE	ETHER- AND ALKALI-SOLUBLE AMINE	WATER-SOLUBLE, HEAT-STABLE AMINE	WATER-SOLUBLE, HEAT-LABILE AMINE
mg/kg/day		*mg/kg body weight*				
		Urinary metabolites of 2 AAF				
20	5	0.35 ± 0.06 [2]	0.59 ± 0.09	0.23 ± 0.01	0.51 ± 0.03	0
0	5	0.25 ± 0.01	0.01 ± 0.01	0.22 ± 0.01	0.36 ± 0.01	0.11 ± 0.02
		Urinary metabolites of 2 AF				
20	5	0.41 ± 0.05	0.36 ± 0.07	0.25 ± 0.01	0.54 ± 0.01	0.43 ± 0.20
0	5	0.34 ± 0.03	0.006 ± 0.004	0.27 ± 0.02	0.51 ± 0.02	1.32 ± 0.04

[1] Dosage of carcinogens = 30 mg/kg body weight.

[2] Standard error.

This argument may account for the smaller excretion of heat-labile amine when the dogs were fed the acetylated rather than when they were fed the free amine. Feeding 20 mg pantothenate/kg/day, an amount 100 times that considered adequate for growth, had a marked effect both upon conjugation and excretion of the heat-labile derivatives, another example of the possible value of feeding at times more than the so-called normal amounts of dietary constituents.

The data in tables 3 and 4 support qualitatively but not quantitatively the increased excretion of conjugated 2-aminofluorene associated with a high riboflavin and pantothenate intake previously reported (Allison and Wase, '52), but these data also emphasize the errors that may be involved in the type of analyses used by these authors to measure degree of conjugation. The presence, for example, of heat-labile diazotizable amines makes impossible an accurate determination of conjugation by diazotizing before and after acid hydrolysis, unless these heat-labile compounds are first removed. The data in tables 3 and 4 suggest an increasing polarity of 2-aminofluorene from ether-, to butanol-, to water-soluble derivatives. The determination of conjugation in these series of amines was limited to the ether-soluble fraction identified spectrophotometrically as 2-acetylaminofluorene.

SUMMARY

The following diazotizable amines were found in the urine of dogs fed 2-acetylaminofluorene or 2-aminofluorene: (a) an ether-soluble, acid-soluble amine, identified spectrophotometrically as 2-aminofluorene; (b) an ether-soluble, conjugated amine identified spectrophotometrically as 2-acetylaminofluorene; (c) an ether-soluble, alkali-soluble amine; (d) an ether-insoluble, water-soluble, heat-stable amine; and (e) an ether-insoluble, water-soluble, heat-labile amine.

Increasing amounts of dietary casein and riboflavin raised the urinary excretion of ether-soluble free and conjugated amines (a) and (b) but decreased the excretion of the water-soluble, heat-labile amine (e).

A high pantothenate intake raised the excretion of conjugated 2-aminofluorene and decreased the excretion of the heat-labile amine (c).

Maximum excretion of free and conjugated aminofluorene and minimum excretion of the heat-labile amine, especially in dogs fed the free amine, were obtained when the protein content of the diet was much above the amount needed for maintenance of an average adult. Similar effects were produced by increasing the amounts of riboflavin and pantothenate, forming diets which experience had demonstrated to be somewhat protective against the toxicity of the aminofluorenes. It is suggested that these diets promote increased oxidation of the carcinogen.

These studies demonstrate that the degree of conjugation of these amines cannot be estimated by determining the degree of diazotization before and after hydrolysis of a urine solution.

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STUDIES ON THE NUTRITIONAL REQUIREMENTS OF CHINCHILLAS [1]

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FOUR FIGURES

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INTRODUCTION

The chinchilla was practically unknown in North America until 1923, when a few animals were brought to this country by an American mining engineer returning from the Andes in South America. Today, 30 years later, it is estimated that there are over 100,000 chinchillas in the United States. In spite of the rapid increase in numbers, there is little scientific information available on this South American rodent. Nutritional requirements have not been established and comparatively little is known about breeding and pathological conditions. King and Orcutt ('52) reported on the ascorbic acid and thiamine requirements but considered their findings tentative due to the limited number of animals used and the lack of uniformity in performance of the animals.

Since the dietary requirement of the chinchilla is a practically unexplored field, our immediate aim was to devise a synthetic diet of known composition which would enable young chinchillas to grow to healthy maturity and which would maintain the weight and health of mature animals. When such a synthetic diet was shown to be adequate, we proposed to use accepted procedures for altering various

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Chinchilla Breeders of America, Inc., Salt Lake City, Utah.

components in order to determine vitamin, protein, fat, carbohydrate, mineral and roughage requirements. However, after 8 months' work, an epidemic bacterial enteritis (to be reported on elsewhere) broke out among our animals. The epidemic prevented us from proceeding with our proposed nutritional program beyond the point of providing a complete synthetic diet adequate for further nutritional work with healthy chinchillas. While our findings were ultimately complicated by a disease factor, the data presented herewith are for apparently healthy animals and are for periods prior to the outbreak of infection, except as noted.

EXPERIMENTAL

General care of animals

Initially we had 67 chinchillas ranging in age from two to 9 months. The animals had been received from various parts of the United States, were all reportedly healthy, and presented a good appearance. The animals were housed individually, some in metal cages $15'' \times 29'' \times 18''$, and some in wooden cages measuring $10'' \times 20'' \times 20''$. All cages were floored with metal screen so that the droppings fell through the openings onto trays of wood shavings. Animals not provided with ready-made nest boxes were supplied with tin fruit cans of a volume of three quarts, which served as combination sleeping quarters and dust baths. The cans were open on one end and rigged with pieces of supporting metal to hold them stationary. Dust baths were given once or twice a week by putting into each can a level tablespoonful of fuller's earth containing 0.1 to 1.0% Spergon [2] fungicide. Later, when there was evidence of eye infections, a generous portion of sulfathiazole was added to the mixture.

The temperature was kept as close to 68° F. as possible, although it could not always be maintained constant. Ac-

[2] We are indebted to the United States Rubber Company, Naugatuck, Conn., for supplies of purified Spergon.

cording to Stubbs ('50) these animals do best at temperatures between 50 and 60°F. Low temperatures produce a better fur, although it is necessary to keep the temperature above freezing. We compromised at 68° to accommodate other animals present in the laboratory. Each chinchilla was provided with its own feeding bowl, inverted water bottle and a small

TABLE 1

Composition of basal diet

CONSTITUENTS	AMOUNT	VITAMIN MIX [5]	
	%		*mg/100 gm ration*
Sucrose	37.0	Thiamine	1.0
Casein, vitamin-free [1]	30.0	Riboflavin	1.4
Cottonseed oil	4.0	Pyridoxine	1.0
Salts IV [2]	4.0	Biotin	0.04
Vitamin mix	2.0	Folic acid	0.3
Potassium acetate	2.5	Menadione	0.2
Magnesium oxide	0.5	Calcium pantothenate	3.0
Gum arabic [3]		Niacin	10.0
or cellulose [4]	20.0	*p*-Aminobenzoic acid	10.0
		Inositol	200.0
		Choline	300.0
		α-Tocopherol acetate	12.0
		β-Carotene	1.2
		D_3 (Calciferol)	8.0 μg
		Sucrose	2.75 gm

[1] General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] J. Biol. Chem., *138:* 459 (1941).

[3] Powdered select gum acacia, U.S.P., S. B. Penick and Co., Chicago, Ill.

[4] Solka-Floc, Brown Company, Berlin, N. H.

[5] We are indebted to Merck and Co., Rahway, N. J., for crystalline vitamins.

block of hard wood to chew on as compensation for the soft diet. The diets were weighed out every morning and food consumption records were kept so that each animal was given just the amount, or a slight excess over the amount, it would consume. The animals were weighed weekly. Sick or ailing animals were examined by a veterinarian and treated in accordance with his directions.

Diets

Since the chinchilla is an herbivorous rodent, the composition of our rations was based on the work of Cannon et al. ('46), Booth et al. ('49), Roine et al. ('49) and Roine and Elvehjem ('50) in connection with guinea pig diets. Both species are anatomically similar and are distinguished by the possession of an unusually large functioning cecum. The basal diet (table 1) contained all the known vitamins, with the exception of C and B_{12}.

Since it has been found that guinea pigs grow better on diets containing a high magnesium and potassium content

TABLE 2

Composition of experimental diets

DIET	CONSTITUENTS
1	Basal including 20% cellulose
2	Basal including 20% gum arabic
3	Basal including 20% cellulose minus K and Mg [1]
4	Basal including 20% gum arabic minus K and Mg [1]

[1] Salts IV in the basal diet contained the following amounts of Mg and K per kg of salts:

K_2HPO_4	322.5 gm
$MgSO_4 \cdot 7H_2O$	102.0 gm
KI	0.8 gm

and gum arabic as roughage, we varied the chinchilla diets with respect to salt and roughage content in order to determine whether this species would react similarly to the guinea pig.

At the start of the experiment all 67 chinchillas were fed the control ration, ground pellets.[3] The animals were shifted to the synthetic ration gradually, depending on acceptance, measured amounts of purified ration being mixed in with the control ration. Appetite and weight gain determined the rapidity with which the animals were put on wholly purified rations. During a short preliminary period gum arabic was

[3] Initially we fed Garver's Rabbit Pellets, Garver's Supply Co., Madison. Later we fed Fromm Chinchilla Pellets, Federal Foods, Inc., Thiensville, Wis.

supplied to some animals at a level of 15%, while others received a combination consisting of 10% cellulose plus 10% gum arabic. These diets were used for variable short periods and since no significant changes were noted on either, they

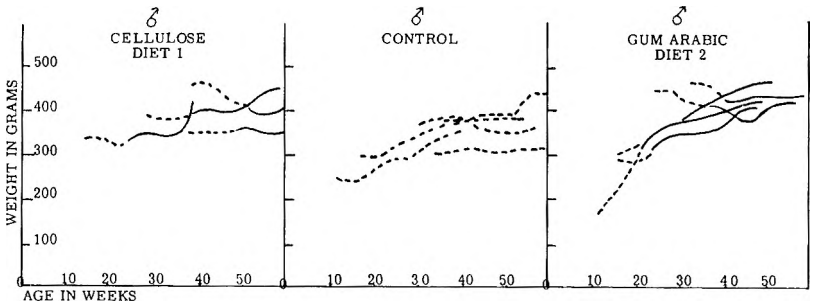

Fig. 1 Comparison of growth of male chinchillas on synthetic and control diets. Dotted line = pellet ration in case of the controls, and partial synthetic plus partial pellets in case of animals on purified diets. Solid line = 100% synthetic.

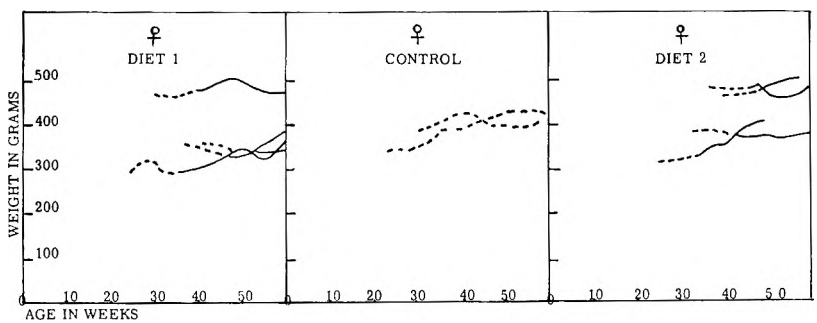

Fig. 2 Comparison of growth of female chinchillas on synthetic and control diets. Dotted line = pellet ration in case of the controls, and partial synthetic plus partial pellets in case of animals on purified diets. Solid line = 100% synthetic.

are not recorded in the figures. The animals were subsequently divided into three dietary groups: a control group (pellets), a cellulose group (diet 1), and a gum arabic group (diet 2; table 2). Results in terms of growth are shown in figures 1 and 2.

After 6 months the animals remaining on diet 1 were divided into two groups approximately equal as to number, sex and

weight. One group was continued on the high salt cellulose diet; the second group was abruptly switched to low salt diet 3. The same procedure was followed with animals on the gum arabic diets. Figure 3 illustrates the growth of representative animals for experimental periods of comparatively short duration because of infection.

When roughage was supplied at a level of 15%, constipation was common among the animals. After the level was raised to 20% some animals continued to be troubled, although

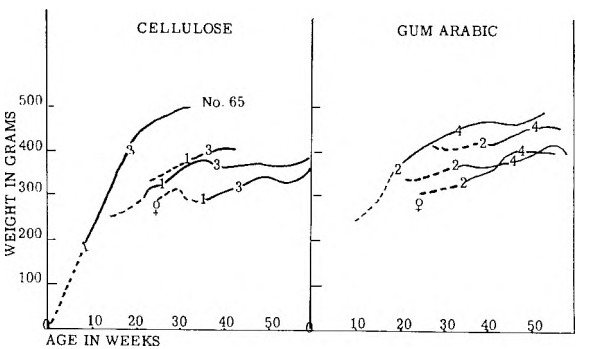

Fig. 3 Growth curves of male and female chinchillas (females indicated) on high and low salt synthetic diets. Dotted line = partial synthetic plus partial pellet ration. Numerals refer to synthetic diets.

others developed diarrhea. The faulty elimination was originally thought to be associated entirely with the enteric infection mentioned earlier. However, some epidemic survivors who were eating gum arabic diets 2 and 4 continued to be troubled with constipation, and those animals exhibiting it to the greatest degree were switched directly to cellulose diets of a corresponding salt content. Results were impressive and are pictured in figure 4.

DISCUSSION RESULTS

The growth curves in figures 1 and 2 show that animals eating the purified diets grew at least as well as the control

group, and that growth on the two types of experimental roughage was analogous. Most of the animals began to level off in weight around 35 to 45 weeks of age, and the range in weight at maturity is seen to be from 320 to 460 gm for males and 350 to 500 gm for females. The rather large

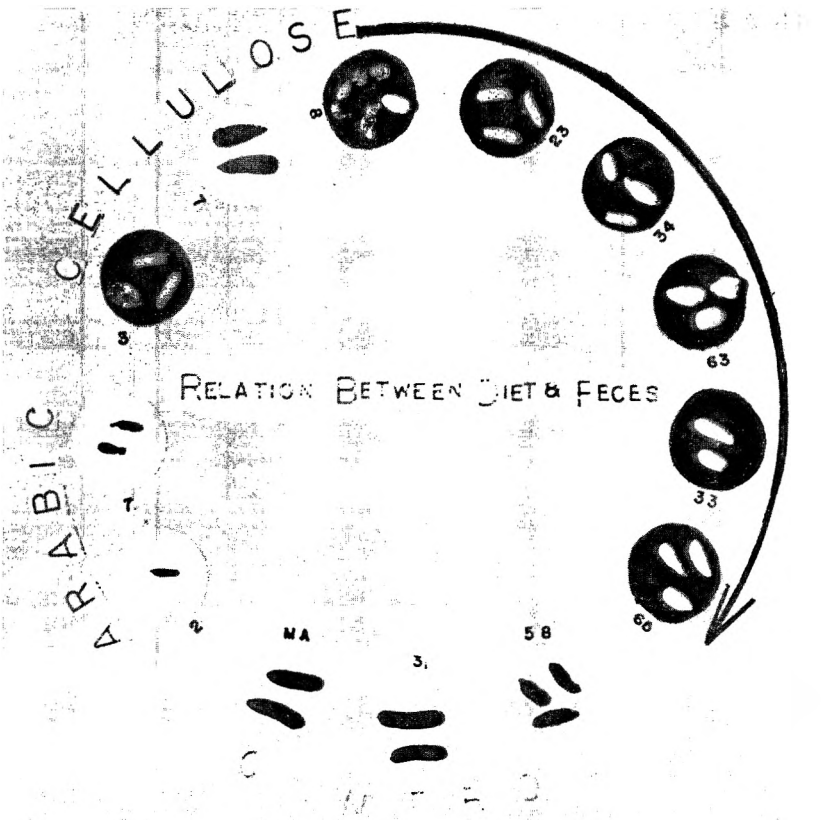

Fig. 4 Relation between diet and feces.

variation in weights within a group may have been due to genetic heterogeneity, or individual idiosyncrasies concerning acceptance of the diet, or environment. No serviceable data on the growth of normal ranch animals are available for comparison. The average weight of normal adult males on a "ranch" diet is reported as 508 gm; adult females weigh

slightly more; 9-week-old weanling males weigh 250 gm, females 232 gm.[4]

Since the slope of the growth curve for younger animals is naturally steeper than for the older ones, the weight data have been segregated and analyzed for three age groups during an experimental period of 21 weeks. Lines of regression have been calculated for the three diets (control, diet 1 and diet 2). In the youngest group (age three to 5 months at the beginning of the experiment), the growth of animals on the purified diets compared favorably with the rate of growth of control animals, the animals on the gum arabic diet showing slightly better gains than the other two groups. In the 6- to 8-month range, the animals receiving the synthetic rations demonstrated somewhat better rates of growth than the controls. The only age group in which the controls exhibited a rate of growth superior to that of animals eating the synthetic diets was the 9- to 11-month range. This may have been due to the poor acceptance of a purified diet by older chinchillas, since there appears to be a linear relationship between consumption and growth. The two controls in this latter group ate an average of 13 gm each per day, whereas the two animals on gum arabic ate an average of 11 gm each and the one animal on cellulose ate only 8 gm per day.

Figure 3 illustrates the growth of animals switched directly from high to low salt diets. During the comparatively short experimental periods that these diets were fed, the animals maintained or made slight gains in weight. Most of the animals were mature at the time they were introduced to the low salt diets, so that the general leveling off in weight was probably due to age. Animal 65 is an exception, being the only weanling on experiment. This animal was born in our laboratory of healthy parents brought into the laboratory 7 months later than the original stock and at a time when the prevailing conditions were more favorable from the point of

[4] Mimeographed report SP1052F, Purina Chinchilla Program, Ralston Purina Company, St. Louis, Mo.

view of temperature control. The first 7 weeks of his growth curve represent the period before weaning. At that time he was put on a mixture of half synthetic diet and half pellets and at 8 weeks on wholly synthetic diet 1. This animal readily accepted the synthetic ration and ate an average of 19 or 20 gm daily. At 18½ weeks he was switched to diet 3. While the gains were not as spectacular on the latter diet as when magnesium and potassium were included at a higher per cent, leveling off was probably due to approaching maturity. This animal died at 35 weeks of age showing typical "epidemic" symptoms, although the epidemic had long since ended.

Fecal droppings relating to the several diets are pictured in figure 4. The number of pellets shown in each circlet represents the relative amount of fecal material produced. The circlets under "Arabic," Nos. 2 and 7, show typical pellets of animals on gum arabic diets 2 and 4. Animals 3, 7, 8, 23 and 34 were originally on gum arabic diets, at which time their pellets were similar to those shown under "Arabic." These animals were shifted abruptly to cellulose diets and their droppings changed in amount and appearance to those shown, the changes taking place in three to 4 days. Animal 8 had produced no pellets for a week prior to the change in diet. Dosages of milk of magnesia and epsom salts elicited no response. After being changed to the cellulose diet, constipation disappeared completely and a great quantity of fecal material was found in the cage. Blood was present in the pellets, indicating intestinal damage due to the disease mentioned earlier. Constipation was not noted in any of these animals placed on cellulose diets, although abnormalities in fecal amount and character occurred at or near death. Animals 63, 33 and 65 were put on cellulose diets originally and their pellets were always approximately as shown. "Control" shows the droppings of animals fed chinchilla pellets. Animal 58 was a gravely sick animal and died just after this picture was taken. Its droppings appeared to contain undigested material and were not as well formed as those of the other

two controls. The droppings of animals 31 and MA had the same appearance. Animal 31 was fed only pellets. Animal MA was fed a more varied diet, consisting mainly of pellets but occasionally supplemented with alfalfa, fresh raw vegetables, nuts and raisins.

SUMMARY

Four synthetic diets, containing all the known vitamins except C and B_{12} but varying in roughage and salt content, have been used in chinchilla nutrition studies. The diets containing gum arabic or cellulose at a level of 20%, plus added amounts of potassium and magnesium, supported apparently normal growth of both young and mature chinchillas for periods of 15 weeks or longer. Reductions in the potassium and magnesium contents of these diets had no discernible effects during the shorter experimental periods employed.

The diets containing cellulose at a level of 20% were physiologically superior to diets containing the same level of gum arabic as roughage.

Although the chinchilla is closely related to the guinea pig, ascorbic acid does not appear to be a dietary essential for this species.

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PROTECTIVE EFFECTS OF LIVER IN IMMATURE RATS FED TOXIC DOSES OF THIOURACIL [1]

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Available data indicate that in addition to the known nutrients, substances are present in our diet which may be required in increased amounts during conditions of stress. Such factors are apparently dispensable under normal conditions, or the requirements for them are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animal's own tissues. Certain drugs or other stressor agents may, however, increase requirements for these substances to such an extent that deficiencies occur, manifested by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient (Ershoff, '48a, '51a). Whole liver is a potent source of such unknown nutrients. Thus, as far back as 1926 Sato reported the presence of a "detoxicating hormone (yakriton)" in water-soluble extracts of liver, a report which was followed by over 100 papers from Sato's laboratory alone on the therapeutic effects of this material in experimental animals and man. Whole liver, or fractions thereof, has been shown to counteract the deleterious effects of massive doses of strychnine (Battelli, '40), sulfanilamide (Chamelin and Funk, '43), promin (Hig-

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gins, '44; Ershoff and McWilliams, '49), atabrine (Ershoff, '48b, '50a) and dinitrophenol (Ershoff, '50b). Similar results have been observed following the administration of toxic doses of diethylstilbestrol (Chamelin and Funk, '43), alpha-estradiol (Ershoff and McWilliams, '48a), desiccated thyroid (Ershoff, '47a, b; Betheil et al., '47), thyroxin, thyroglobulin and iodinated casein (Ershoff and McWilliams, '48b) and cortisone acetate (Ershoff, '51b, '53a).

In the case of at least several of these drugs (thyroid, thyroxin, thyroglobulin, iodinated casein, atabrine, promin and cortisone acetate), a protective factor is retained in the water-insoluble fraction of liver, with little if any activity in the water-soluble extract. It would appear, therefore, that liver contains at least two factors, apparently distinct from any of the known nutrients, whose requirements may be increased under conditions of stress. In view of the protective effects of liver in animals administered toxic doses of the drugs indicated above, experiments were undertaken to determine the effects of liver feeding on immature rats administered toxic doses of thiouracil.

PROCEDURE AND RESULTS

Experiment 1. Comparative effects of desiccated whole liver, casein and the known B vitamins on the growth increment and thyroid weight of immature rats fed massive doses of thiouracil

The basal ration employed in the present experiment consisted of sucrose, 61%; casein,[2] 24%; salt mixture,[3] 5%; cottonseed oil,[4] 8%; wheat germ oil,[5] 2%; and the following synthetic vitamins per kilogram of diet: thiamine hydrochlo-

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

[3] Hubbell, Mendel and Wakeman salt mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Wesson.

[5] VioBin.

ride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; 2-methyl-naphthoquinone, 10 mg; and choline chloride, 2 gm. To each kilogram of diet were also added 4,000 U.S.P. units of vitamin A [6] and 400 U.S.P. units of vitamin D.[7] The vitamins were added in place of an equal amount of sucrose. In addition to the basal ration the following diets were also employed: (1) basal ration plus the following additional vitamin supplements per kilogram of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; biotin, 5 mg; folic acid, 10 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; and vitamin B_{12}, 150 μg; (2) basal ration plus 10% whole liver powder [8]; and (3) basal ration plus 10% casein.[9] Thiouracil was incorporated in the above diets at levels of 0.1, 0.25 and 0.5%. The vitamin supplements, thiouracil, liver and casein, were added in place of an equal amount of sucrose. In addition to the above diets, a control ration was also tested, which consisted of the basal ration with thiouracil omitted.

One hundred and four male rats of the Long-Evans strain were selected at 21 to 24 days of age and at a body weight of 40 to 52 gm for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets ad libitum (8 rats per group). Diets were made up weekly and stored under refrigeration. Rats were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. After 42 days of feeding, animals were autopsied and thyroid weight was determined. Results are summarized in table 1.

All rats survived the experimental period of 6 weeks. Findings indicate that the addition of thiouracil to the basal ration caused a significant retardation in growth and a marked

[6] Crystalets, Chas. Pfizer and Co., New York, N. Y.

[7] HY-DEE Powder, Standard Brands, New York, N. Y.

[8] Desiccated liver N.F. (lot L 38412), Armour and Co., Chicago, Illinois.

[9] See footnote 2, p. 438.

increase in thyroid weight. Supplements of the known B vitamins or of hot alcohol-extracted casein at a 10% level in the diet were without significant effect on either rate of growth or degree of thyroid hyperplasia. Liver,[10] however, when fed at a 10% level in the diet, restored the growth increment of

TABLE 1

Comparative effects of desiccated whole liver, casein and the known B vitamins on the growth increment and thyroid weight of immature male rats fed massive doses of thiouracil

(8 animals per group)

SUPPLEMENTS FED WITH BASAL RATION	THIOURACIL IN RATION	INITIAL BODY WT.	GAIN IN BODY WT.[1,2]	THYROID WT.	
				Ave.	Range
	%	gm	gm	mg	
None	0.0	47.3	211.8 ± 8.1	16	11– 20
None	0.1	48.1	87.4 ± 6.2	49	36– 64
B vitamins	0.1	47.0	78.3 ± 4.8	43	31– 52
Whole liver powder [3]	0.1	47.5	187.2 ± 8.4	7	6– 8
Casein	0.1	46.1	108.9 ± 10.3	39	31– 54
None	0.25	46.4	63.6 ± 3.5	73	46–127
B vitamins	0.25	47.8	67.0 ± 0.9	71	52– 88
Whole liver powder [3]	0.25	45.9	183.9 ± 8.8	8	74– 94
Casein	0.25	46.1	84.2 ± 4.3	54	44– 69
None	0.5	47.2	45.4 ± 4.6	50	30– 73
B vitamins	0.5	48.0	71.0 ± 4.4	76	41–160
Whole liver powder [3]	0.5	47.2	172.6 ± 13.9	8	7– 9
Casein	0.5	47.4	75.4 ± 4.3	58	38– 83

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

[2] Experimental period, 42 days.

[3] Armour's desiccated liver N.F. (lot L 38412).

thiouracil-fed rats to virtually normal levels and not only prevented thyroid hyperplasia but actually resulted in thyroid glands which were 50% smaller than those in control rats on the thiouracil-free basal ration. The above findings occurred on all three levels of thiouracil intake.

At the conclusion of the above experiment 48 male and 66 female rats of the Long-Evans strain were selected at 21 to

[10] See footnote 8, p. 439.

23 days of age and at a body weight of 38 to 50 gm and were fed the following diets (8 male and 6 female rats per group): (1) basal ration; (2) basal ration plus 0.25% thiouracil; (3) basal ration plus 0.25% thiouracil plus the B vitamin supplement previously described; (4) basal ration plus 0.25%

TABLE 2

Variations in the activity of commercial sources of desiccated liver on the growth increment and thyroid weight of immature rats fed massive doses of thiouracil

SUPPLEMENTS FED WITH BASAL RATION	THIOURACIL IN RATION	NUMBER OF ANIMALS	INITIAL BODY WT.	GAIN IN BODY WT.[1,2]	THYROID WT.	
					Ave.	Range
	%		*gm*	*gm*	*mg*	
		Males				
None	0.0	8	42.2	129 ± 4.9	14	10– 18
None	0.25	8	42.8	57 ± 2.8	43	33– 56
B vitamins	0.25	8	42.7	74 ± 2.5	34	26– 47
Armour's desiccated liver N.F.[3]	0.25	8	42.4	135 ± 6.3	7	6– 8
Wilson's desiccated liver N.F.[4]	0.25	8	43.0	76 ± 2.6	68	55– 76
VioBin's liver powder[5]	0.25	8	42.6	84 ± 3.8	64	60– 72
		Females				
None	0.0	6	42.0	98 ± 2.8	12	10– 15
None	0.25	6	43.4	45 ± 2.2	38	29– 46
B vitamins	0.25	6	42.8	56 ± 2.4	33	27– 40
Armour's desiccated liver N.F.[3]	0.25	6	41.7	94 ± 3.2	8	7– 10
Wilson's desiccated liver N.F.[4]	0.25	6	43.0	47 ± 2.3	66	52– 74
VioBin's liver powder[5]	0.25	6	41.8	54 ± 3.3	53	49– 60
None	0.5	6	42.8	41 ± 4.9	56	42– 64
B vitamins	0.5	6	42.8	47 ± 3.9	51	46– 58
Armour's desiccated liver N.F.[3]	0.5	6	42.6	90 ± 4.3	8	7– 10
Wilson's desiccated liver N.F.[4]	0.5	6	42.7	52 ± 3.2	84	53–110
VioBin's liver powder[5]	0.5	6	43.0	54 ± 2.0	56	40– 68

[1] Including standard error of the mean. See footnote 1, table 1.

[2] Experimental period, 28 days.

[3] Armour's desiccated liver N.F. (lot L 38412).

[4] Wilson's desiccated liver N.F. (lot 83479).

[5] VioBin's liver powder (lot L-40-59).

thiouracil plus 10% liver [11]; (5) basal ration plus 0.25% thiouracil plus 10% liver [12]; and (6) basal ration plus 0.25% thiouracil plus 10% liver powder.[13] In addition, experiments were also conducted with 6 female rats per group fed the same thiouracil-containing diets indicated above but with the thiouracil content of the diet raised to 0.5% of the ration. The experimental procedure was similar to that employed in the first experiment. Animals were autopsied after 28 days of feeding and thyroid weights determined. Results are summarized in table 2.

In agreement with the previous experiment, thiouracil when incorporated at a 0.25% level in the basal ration caused a significant retardation in growth and increase in thyroid weight as compared to that observed in rats fed a similar diet with thiouracil omitted. As in the previous experiment, supplements of the known B vitamins were without significant effect on either rate of growth or degree of thyroid hyperplasia. Desiccated liver,[14] however, when fed at a 10% level in the diet restored the growth increment of thiouracil-fed rats to virtually normal levels and not only prevented thyroid hyperplasia but resulted in thyroid glands which were smaller than those in control rats fed the thiouracil-free basal ration. The above findings occurred both in male and female rats. Similar results were obtained with female rats fed diets containing 0.5% thiouracil. In contrast to the results obtained with this lot of desiccated liver, however, another lot [15] and liver powder,[16] when fed under comparable conditions, had little if any effect in counteracting the growth retardation or thyroid enlargement of immature rats (of both sexes) fed toxic doses of thiouracil under conditions of the present experiment.

[11] See footnote 8, p. 439.

[12] Desiccated liver N.F. (lot 83479), Wilson Laboratories, Chicago, Illinois.

[13] Liver powder, desiccated and defatted at 37°C., VioBin Corporation, Monticello, Illinois.

[14] See footnote 8, p. 439.

[15] See footnote 12.

[16] See footnote 13.

Experiment 2. Variations in the activity of commercial batches of desiccated liver N.F. and liver residue on the body and thyroid weights of immature rats fed toxic doses of thiouracil

In view of the differential effects of the three batches of liver in experiment 1 on the growth increment and thyroid weight of immature rats fed toxic doses of thiouracil, tests were made of the anti-thiouracil activity of other batches of desiccated liver N.F. Similar studies were also conducted with liver residue [17] and water-soluble liver concentrate. One hundred and twenty-eight male rats of the Long-Evans strain were selected at 21 to 24 days of age and at a body weight of 40 to 52 gm and were fed the following diets (8 animals per group): (1) basal ration; (2) basal ration plus 0.25% thiouracil; (3) basal ration plus 0.25% thiouracil plus the B vitamin supplement previously described; and (4) basal ration plus 0.25% thiouracil plus the liver supplements indicated in table 3. The desiccated liver N.F. and liver residue supplements were incorporated at a 10% level in the diet; the liver concentrate fractions at a 2.5% level. The various supplements were added in place of an equal amount of sucrose. Feeding was continued for 42 days. Rats were then autopsied and thyroid weight determined. Results are summarized in table 3.

Findings indicate that the various liver supplements differed significantly in their effect on the body and thyroid weights of immature male rats fed toxic doses of thiouracil. In agreement with the previous experiments, thiouracil when incorporated at a 0.25% level in the basal ration caused a significant retardation in growth and increase in thyroid weight as compared to those observed in rats fed a similar diet with thiouracil omitted. Supplements of the known B vitamins as reported previously were without significant effect on either rate of growth or degree of thyroid hyper-

[17] This liver fraction consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

TABLE 3

Variations in the activity of commercial batches of desiccated liver N.F. and liver residue on the body and thyroid weights of immature rats fed toxic doses of thiouracil

(8 animals per group)

SUPPLEMENTS FED WITH BASAL RATION	THIOURACIL IN RATION	INITIAL BODY WT.	GAIN IN BODY WT.[1,2]	THYROID WT.	
				Ave.[1]	Range
	%	gm	gm	mg	
None	0.0	44.8	185 ± 7.1	16 ± 1.6	12– 21
None	0.25	44.9	57 ± 4.7	60 ± 3.6	54– 75
B vitamins	0.25	46.1	59 ± 3.6	65 ± 4.4	41– 76
Desiccated liver N.F. (Armour, lot L 38412)	0.25	44.5	189 ± 8.2	8 ± 0.6	7– 10
Desiccated liver N.F. (Armour, lot L 35301)	0.25	45.5	166 ± 7.1	107 ± 11.3	52–148
Desiccated liver N.F. (Armour, lot K 33510)	0.25	45.6	111 ± 5.2	80 ± 6.4	47–103
Desiccated liver N.F. (Armour, lot K 33810)	0.25	45.6	83 ± 5.6	96 ± 11.7	63–173
Desiccated liver N.F. (Wilson, lot 33479)	0.25	44.6	62 ± 3.5	55 ± 4.6	33– 80
Desiccated liver N.F. (Wilson, lot 37551)	0.25	45.4	81 ± 3.0	66 ± 7.6	40– 98
Desiccated liver N.F. (Wilson, lot 37656)	0.25	45.9	71 ± 4.5	63 ± 6.5	36– 90
Liver residue (Armour, lot L 31303)	0.25	44.6	184 ± 7.1	58 ± 6.0	28– 81
Liver residue (Wilson, lot 33975)	0.25	44.9	88 ± 5.4	69 ± 8.5	57– 98
Liver concentrate (Armour, lot 13204)	0.25	45.5	75 ± 7.0	57 ± 3.9	41– 79
Liver concentrate (Wilson, lot 86456)	0.25	45.7	79 ± 5.0	73 ± 8.7	52–100
Liver residue (Armour, lot L 31303) + Liver concentrate (Armour, lot L 13204)	0.25	44.6	198 ± 9.5	110 ± 19.6	53–216

[1] Including standard error of the mean. See footnote 1, table 1.

[2] Experimental period, 42 days.

plasia. Two out of 7 samples of desiccated liver N.F.,[18] however, restored the growth increment of thiouracil-fed rats to virtually normal levels, whereas a third sample[19] significantly increased the growth increment over that observed in rats fed a similar diet with liver omitted. The remaining 4 samples of desiccated liver N.F.[20] had little if any growth-promoting activity. Of two samples of hog liver residue which were tested, one[21] was very active, while the other[22] was virtually devoid of activity. Two samples of hog liver concentrate[23] were both inactive. No correlation was observed between the growth-promoting and the thyroid-hyperplasia-preventing effects of the liver supplements. Desiccated liver N.F.,[24] in agreement with previous findings, not only counteracted the increase in thyroid weight but resulted in thyroid glands which were smaller than those in control rats fed the thiouracil-free basal ration. All other liver supplements tested were ineffective in preventing thyroid hyperplasia in thiouracil-fed rats; and at least one of them[25] resulted in thyroid glands which were significantly *larger* than those of rats fed the basal ration plus 0.25% thiouracil. An unexpected finding was the observation that whereas the thyroid glands of rats fed either Armour's liver residue or Armour's liver concentrate as a supplement did not differ significantly from those of rats fed the basal ration plus 0.25% thiouracil, a marked increase in thyroid weight occurred in rats fed both these supplements. In view of the large standard deviation in the latter series, however, it is questionable whether the increase in thyroid weight of rats in the latter group was statistically significant.

[18] Armour's lot L 38412, derived from hog liver, and Armour's lot L 35301, derived from beef liver.

[19] Armour's lot K 33510, derived from hog liver.

[20] One from Armour and three from Wilson, all derived from hog liver.

[21] Armour's lot L 31303.

[22] Wilson's lot 83975.

[23] Armour's lot L 13204 and Wilson's lot 86456.

[24] See footnote 8, p. 439.

[25] Armour's desiccated liver N.F., lot L 35301.

Experiment 3. On the correlation between the iodine content of liver and its growth-promoting activity in immature rats fed toxic doses of thiouracil

It is well-established that thyroxin will counteract the growth retardation and thyroid hyperplasia of immature rats fed massive doses of thiouracil. Inasmuch as comparable results were obtained with at least one of the liver supplements tested in experiment 2,[26] the possibility arose that the protective effect of liver may have been due to its content of thyroactive material. Various liver supplements tested in experiment 2 were accordingly analyzed for their total iodine content in an effort to determine whether the growth-promoting or thyroid weight-inhibiting properties of these supplements were correlated with their iodine content. Total and inorganic iodine were determined by the Chaney method (Chaney, '40, '50) with slight modifications as required to make the procedure applicable to liver.[27] Results are summarized in table 4, together with the data (obtained in experiment 2) on the effects of these supplements on growth increment and thyroid weight of immature male rats fed toxic doses of thiouracil.

Findings indicate that the various liver supplements differed significantly in iodine content, ranging from 15 to 1,155 μg of total iodine per 100 gm of sample, of which only 3 to 6% was inorganic iodine. The data suggest that the growth-promoting activity of liver in immature rats fed toxic doses of thiouracil was correlated with its iodine content, being greatest in the three groups with the highest iodine content. Considerable variation was observed, however, in the iodine content of these supplements, since they ranged from 62 to 1,155 μg of total iodine per 100 gm of sample. All other liver supplements tested had less than 35 μg of total iodine per

[26] See footnote 8, p. 439.

[27] The iodine determinations were made by Dr. A. L. Chaney of the Albert L. Chaney Chemical Laboratory, Glendale, Calif. The technical services of Dr. Chaney are gratefully acknowledged.

100 gm of sample and exhibited a markedly reduced growth-promoting activity. Desiccated liver N.F.,[28] which was the only liver supplement tested that prevented thyroid hyperplasia in thiouracil-fed rats, had the highest total iodine content of any of the liver samples. The total iodine content of this material (1,155 μg per 100 gm of sample) was significantly greater than that of the other supplements. No correlation was observed among the remaining samples between iodine content and thyroid weight.

TABLE 4

Total iodine content of liver and its relationship to the growth increment and thyroid weight of immature rats fed toxic doses of thiouracil

LIVER SUPPLEMENT	GAIN IN BODY WT.[1,2]	THYROID WT.[1]	TOTAL IODINE CONTENT OF SUPPLEMENT	
			μg/100 gm of supplement	μg/kg of ration
	gm	*mg*		
Desiccated liver N.F. (Armour, lot L 38412)	189 ± 8.2	8 ± 0.6	1,155	1,155
Desiccated liver N.F. (Armour, lot L 35301)	166 ± 7.1	107 ± 11.3	62	62
Desiccated liver N.F. (Armour, lot K 33510)	111 ± 5.2	80 ± 6.4	34	34
Desiccated liver N.F. (Armour, lot K 33810)	83 ± 5.6	96 ± 11.7	24	24
Desiccated liver N.F. (Wilson, lot 83479)	62 ± 3.5	55 ± 4.6	15	15
Desiccated liver N.F. (Wilson, lot 87656)	71 ± 4.5	63 ± 6.5	26	26
Liver residue (Armour, lot L 31303)	184 ± 7.1	58 ± 6.0	347	347
Liver residue (Wilson, lot 83975)	88 ± 5.4	69 ± 8.5	18	18

[1] Including standard error of the mean. See footnote 1, table 1.

[2] Experimental period, 42 days.

[28] See footnote 8, p. 439.

Experiment 4. Effects of graded doses of desiccated thyroid and kelp on the growth increment and thyroid weight of immature rats fed toxic doses of thiouracil

The purpose of the following experiment was to determine the effects of graded doses of desiccated thyroid on the growth increment and thyroid weight of immature rats fed toxic doses of thiouracil. Tests were also conducted with animals fed desiccated kelp (Macrocystis pyrifera),[29] as a source of organically bound iodine. The experimental procedure was similar to that employed in the first experiment. Fifty-six male rats of the Long-Evans strain were selected at 21 to 23 days of age and at a body weight of 38 to 50 gm and were fed the following diets (8 animals per group): (1) basal ration; (2) basal ration plus 0.25% thiouracil; (3) basal ration plus 0.25% thiouracil plus 500 mg desiccated thyroid[30] per kilogram of diet; (4) basal ration plus 0.25% thiouracil plus 250 mg desiccated thyroid per kilogram of diet; (5) basal ration plus 0.25% thiouracil plus 125 mg desiccated thyroid per kilogram of diet; (6) basal ration plus 0.25% thiouracil plus 62.5 mg desiccated thyroid per kilogram of diet; and (7) basal ration plus 0.25% thiouracil plus 10% kelp. The desiccated thyroid and kelp were added in place of an equal amount of sucrose. Feeding was continued for 42 days. Rats were then autopsied and thyroid weight determined. Results are summarized in table 5.

In agreement with earlier findings, thiouracil when incorporated at a 0.25% level in the basal ration caused a significant retardation in growth and increase in thyroid weight as compared to those observed in rats fed a similar diet with thiouracil omitted. Desiccated thyroid when fed at a level of 500 mg per kilogram of diet restored the growth increment of thiouracil-fed rats to normal and not only prevented thy-

[29] Desiccated kelp, Emory W. Thurston Laboratories, Los Angeles, Calif. This material contained 0.122% total iodine of which 81% was water-extractable iodine.

[30] Thyroid powder U.S.P., Armour and Co., Chicago, Illinois.

TABLE 5

Effects of graded doses of desiccated thyroid and kelp on the growth increment and thyroid weight of immature rats fed toxic doses of thiouracil

(8 animals per group)

SUPPLEMENTS FED WITH BASAL RATION	THIOURACIL IN RATION	INITIAL BODY WT.	GAIN IN BODY WT.[1,2]	THYROID WT.[1]		TOTAL IODINE CONTENT OF SUPPLEMENT IN μG/KG OF RATION
				Ave.	Range	
	%	*gm*	*gm*	*mg*		
None	0.0	42.6	176 ± 13.9	15 ± 1.0	12–20	....
None	0.25	42.4	74 ± 3.6	67 ± 5.2	39–85	....
Desiccated thyroid (500 mg/kg of diet)	0.25	42.6	172 ± 11.2	9 ± 0.9	6–14	1,000
Desiccated thyroid (250 mg/kg of diet)	0.25	42.3	144 ± 5.5	26 ± 2.6	16–36	500
Desiccated thyroid (125 mg/kg of diet)	0.25	42.0	134 ± 4.3	66 ± 5.6	34–83	250
Desiccated thyroid (62.5 mg/kg of diet)	0.25	42.7	139 ± 7.7	67 ± 5.7	40–85	125
Kelp (10% of diet)	0.25	43.0	92 ± 4.1	60 ± 7.5	32–84	122,000

[1] Including standard error of the mean. See footnote 1, table 1.

[2] Experimental period, 42 days.

roid hyperplasia but resulted in thyroid glands which were smaller than those in control rats fed the thiouracil-free basal rations. Smaller doses of thyroid (250 mg, 125 mg and 62.5 mg per kilogram of diet) also promoted a significant increment in body weight, although this effect was less marked than that obtained with the higher thyroid dosage. An unexpected finding was the observation that desiccated thyroid in doses of 62.5 mg and 125 mg per kilogram of diet, although effective in promoting growth, was without effect in preventing thyroid hyperplasia in immature rats fed toxic doses of thiouracil. Larger doses of thyroid (250 mg per kilogram of diet or higher), however, did prevent thyroid hyperplasia. These findings suggest that increase in body weight is a more sensitive indicator of thyroid hormone than prevention of thyroid hyperplasia in the immature thiouracil-fed rat. That the protective effects of desiccated thyroid indicated above were due to the thyroid hormone and not to iodine per se (either inorganic or organically bound), is indicated by the fact that the kelp-containing ration which provided more than 100 times the total iodine contained in the highest thyroid dosage was without significant effect on either body or thyroid weight.

DISCUSSION

Present findings indicate that there is present in some batches of commercial desiccated whole liver a factor (or factors) which will counteract the growth retardation and thyroid enlargement of immature rats fed toxic doses of thiouracil. The protective factor is apparently distinct from any of the known B vitamins (including vitamin B_{12}) and is not present in significant amounts in hot alcohol-extracted casein or kelp. Commercial batches of desiccated whole liver, however, vary markedly in their content of the protective factor. Only three of 7 batches of desiccated liver N.F. had significant growth-promoting activity and only one of the 7 samples tested prevented thyroid hypertrophy. The protective factor when present (at least that responsible for the increase in

body weight) is retained in the water-insoluble fraction of liver. Meites ('50) has previously reported that crystalline vitamin B_{12} counteracted the growth retardation and thyroid hypertrophy of immature rats fed a natural food ration containing 0.1% thiouracil. Greer ('51), employing a similar ration, was unable to confirm these results. Present findings indicate that vitamin B_{12} under the conditions of the present experiment was similarly inactive.

It is well-established that thiouracil inhibits thyroid hormone synthesis with a resulting retardation in growth and decrease in BMR that parallels that occurring in surgically thyroidectomized rats (Astwood, '43; Williams et al., '44; Meyer and Ransom, '45; Gordon et al., '46). Associated with these changes, hypertrophy of the thyroid occurs as a result of an increased production of thyrotrophic hormone by the anterior pituitary (MacKenzie and MacKenzie, '43; Astwood et al., '43). The above effects can be counteracted by thyroid substance but not by the administration of iodine per se. Present findings confirm the protective effect of desiccated thyroid in immature rats fed toxic doses of thiouracil. The administration of 500 mg desiccated thyroid per kilogram of diet completely counteracted the growth retardation and thyroid hypertrophy of immature rats fed a purified ration containing 0.25% thiouracil. Smaller doses, although promoting an increase in body weight, did not prevent thyroid hypertrophy. It would appear, therefore, that more than 250 mg of desiccated thyroid per kilogram of diet are required to prevent thyroid hypertrophy under conditions of the present experiment. Inasmuch as desiccated thyroid in doses as small as 62.5 mg per kilogram of diet resulted in a significant increment in body weight, it is apparent that less thyroid hormone is required for gain in body weight than for prevention of thyroid hypertrophy in the immature thiouracil-fed rat.

Present data indicate that commercial batches of desiccated liver N.F. vary markedly in iodine content, ranging from 15 to 1,155 μg of total iodine per 100 gm of sample. Findings

suggest that the growth-promoting activity of liver in immature rats fed toxic doses of thiouracil was correlated with its iodine content, being greatest in the three groups with the highest iodine content. Liver supplements with less than 30 μg of total iodine per 100 gm of sample had no significant growth-promoting activity. It is of interest that the only liver sample that prevented thyroid hypertrophy in immature thiouracil-fed rats was the one with the highest iodine content. These findings suggest that more of the liver factor was required to prevent thyroid hypertrophy than to promote growth, although the possibility has not been excluded that gain in body weight and the prevention of thyroid hypertrophy may be due to different factors.

Present findings indicate that some batches of desiccated whole liver and water-insoluble liver residue contain considerable amounts of thyroid hormone or some other thyroactive material, and that the latter is responsible for the protective effects of these liver batches on immature rats fed toxic doses of thiouracil. On the basis of total iodine supplement per kilogram of diet, liver appears to be somewhat more active than desiccated thyroid as a growth-promoting factor, particularly in ranges below 300 μg of total iodine per kilogram of diet, which suggests that the liver factor is not identical to thyroid hormone. The number of animals in each group, however, was too small to be conclusive on this point. On the basis of the total iodine content of supplements which prevented thyroid hyperplasia, the iodine content of the liver and thyroid-containing rations was virtually identical. The growth-promoting factor in liver residue appears to be distinct from the "antithyrotoxic factor" of liver (Ershoff, '47b, '53b), inasmuch as Wilson's liver residue (lot 83975) is a potent source of "antithyrotoxic factor," whereas this material was virtually inert under the conditions of the present experiment.

No data are available to account for the marked variations in total iodine and thyroactive activity of the liver samples tested in the present experiment.

SUMMARY

Some batches of commercial desiccated whole liver contain a factor (or factors) which will counteract the growth retardation and thyroid hyperplasia of immature rats fed toxic doses of thiouracil. The protective factor is apparently distinct from any of the known B vitamins and is not present in significant amounts in hot alcohol-extracted casein or kelp. Commercial batches of desiccated whole liver vary markedly in their content of this factor or factors. The protective effects of liver are correlated with its iodine content. Data are presented which indicate that some batches of desiccated whole liver and water-insoluble liver residue contain considerable amounts of thyroid hormone or some other thyroactive material and that the latter are responsible for the protective effects of these liver batches on immature rats fed toxic doses of thiouracil.

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THE INFLUENCE OF THE ASH CONTENT OF THE RUMEN INGESTA ON THE HYDROGEN ION CONCENTRATION IN THE BOVINE RUMEN [1]

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TWO FIGURES

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The rumen of the bovine plays an essential and important role in the digestive process, since it is here that cellulose, the main constituent of the crude fiber of plant foods, is utilized. There is a paucity of information, pointing to the fact that bacteria are the chief agencies involved in cellulose digestion. For optimum bacterial activity, the hydrogen ion concentration needs to be within a certain range (Smith, '41).

Numerous workers (Hale et al., '40; Kick et al., '38; Monroe and Perkins, '39; Olson, '41; Schwarz and Stremnitzer, '26; Smith, '41; Stalfors, '26; Villares, '40; Wegner et al., '41) have reported on the pH of rumen ingesta. Kick et al. ('38) found that the reaction was more alkaline on alfalfa hay alone than on a grain-silage-hay ration or a grain-hay ration. It was found that as the grain was increased the pH was lowered. Smith ('41) reported that the addition of beet pulp to an alfalfa hay ration resulted in a lowered pH in the rumen. Hale et al. ('40) reported that the pH values of the rumen contents on an alfalfa hay ration were definitely higher than the pH values obtained on a ration of silage, hay

[1] Research paper 1099, Journal Series, University of Arkansas. Published with the permission of the Director of the Arkansas Agricultural Experiment Station.

and soybean oil meal. Monroe and Perkins ('39) reported a more acid rumen pH when animals were on both bluegrass and alfalfa pasture, as compared to the rumen pH when such roughages as corn or A. I. V. silage and alfalfa hay were fed. Thus, it appears from the above results that the type of feed influences the pH of the rumen.

Another item influencing the pH of the rumen is the saliva. The large amount of carbon dioxide as bicarbonate, together with the high phosphate content, are functionally most important for rendering the fluid an ideal buffer for the bacterial digestion that goes on in the rumen. The quantity of saliva produced per day has been estimated at 56 kg for the ox by Colin (1886) and at 50 l for the cow by Markoff ('13). Zuntz ('13) claimed that the amount of alkali secreted each day in the saliva of the ox must be about 6 times that contained in the blood, and Markoff ('13) calculated that his cow produced as much as 300 to 350 gm of alkali, expressed as sodium bicarbonate. The latter figure may be compared with the value of 329 gm of volatile acid (calculated as acetic acid) in the rumen and reticulum of an ox reported by Elsden et al. ('46). It is evident, then, that the saliva flowing into the rumen each day supplies a large volume of liquid for the suspension of the ingesta and a relatively large amount of buffering material which tends to hold the pH in a relatively narrow range. The literature pertaining to the pH of bovine saliva has been reviewed by Reid and Huffman ('49).

In view of the apparent buffering action of the minerals in the saliva, a question arises as to the possible buffering action of the ash contained in the feed of the animal. Ammerman and Thomas ('52) have reported variations in the buffering capacity of rumen ingesta as affected by the ration. Plant juices of bluegrass were more highly buffered than were the juices from Ladino clover and alfalfa. Also the juices from mature Ladino and alfalfa appeared to be more highly buffered than the juices from these forages when young and succulent.

METHODS

Two Holstein-Friesian yearling steers with rumen fistulas were fed Korean lespedeza, Sericea lespedeza, and upland prairie hays as the only source of feed nutrients. The hay was sampled for chemical analysis before being fed to the animals. A preliminary feeding period of at least two weeks was arranged for each of the hays. This allowed time for the steers to become accustomed to the various roughages and for complete elimination of plant remnants from the rumen. The rumen contents were removed in the morning just before feeding (24 hours following the previous feeding), weighed, mixed, sampled for chemical analysis, and replaced in the rumen. This identical process was again repeated at three, 6, 9, 12 and 24 hours after the animal was fed the roughage. Two replications were obtained from each steer for each of the hays studied.

The pH values were determined on the uncentrifuged liquid expressed from a sample of the ingesta. All pH determinations were made on the fresh material immediately after the samples were taken. The readings were made on the Beckman pH Meter, laboratory model G.

Each sample of rumen contents was then sealed in a glass jar and placed in a deep freeze at — 15°C. The samples were later opened, thawed in a water bath at 40°C., mixed and sampled for chemical analysis. The moisture percentage was determined by the method of Horwitt, Cowgill and Mendel ('36); ether extract and ash were determined by the Association of Official Agricultural Chemists methods ('50).

RESULTS

Data pertaining to the chemical composition of the hays and rumen contents are given in table 1. The pH values are also presented in table 1 and in figure 1.

The pH values follow the general pattern already reported (Smith, '41). The values shown are the average of two repetitions taken a week or more apart and after the animal had

been on the roughage a sufficient length of time to become adjusted to it. Before feeding in the *morning* the reaction *was at or near alkalinity.* After feeding there was a change

TABLE 1

Chemical composition of roughages and rumen contents at various hours after feeding

MATERIAL SAMPLED	LBS. WET MATERIAL IN RUMEN	% DRY MATTER	% ASH	% FAT	% FATTY ACIDS	pH
		Korean lespedeza				
Rumen contents						
0-hour	61.1	12.58	9.43	6.64	1.23	7.0
3-hour	96.3	15.66	8.44	6.40	1.06	6.5
6-hour	86.8	15.06	8.78	4.70	1.29	6.6
9-hour	79.0	17.64	9.16	6.54	.77	6.8
12-hour	71.2	13.45	9.01	4.92	.90	6.8
24-hour	59.8	12.58	9.96	6.06	1.23	7.0
Feed	8.77	89.10	6.89	8.06	1.16	
		Sericea lespedeza				
Rumen contents						
0-hour	62.0	16.1	8.78	5.10	1.37	7.2
3-hour	93.5	19.3	7.90	6.03	1.07	6.7
6-hour	91.7	18.6	7.79	5.17	1.33	6.7
9-hour	83.6	17.6	7.94	7.82	1.31	6.9
12-hour	80.4	16.5	8.17	8.94	1.28	7.0
24-hour	56.1	15.6	9.20	5.08	1.13	7.3
Feed	8.1	91.5	5.17	4.69	1.11	
		Prairie hay				
Rumen contents						
0-hour	74.0	15.63	10.31	3.18	1.16	6.9
3-hour	98.6	16.60	9.86	3.64	1.07	6.6
6-hour	88.0	17.70	9.77	3.81	1.11	6.7
9-hour	84.9	16.08	10.61	2.90	1.03	6.8
12-hour	78.4	16.60	10.21	1.98	1.20	6.9
24-hour	57.5	15.00	10.65	3.49	1.31	6.9
Feed	6.5	91.56	6.73	1.94	.74	

to an acid condition, which reached the low point at approximately three hours after feeding. There was then a gradual upward trend until at the end of 24 hours the reaction was approximately the same as before the animal was fed.

The trend of the reactions shown by the curve (fig. 1) from near neutrality at feeding time to an acid condition at three hours following feeding is undoubtedly due to the production of volatile fatty acids in the rumen during this period, as shown by Phillipson ('42) and McClymont ('51). Volatile fatty acids were not determined in this study, but rather total fatty acids. It has been shown that a large percentage (96.2%) of the total acids are volatile (McClymont, '51).

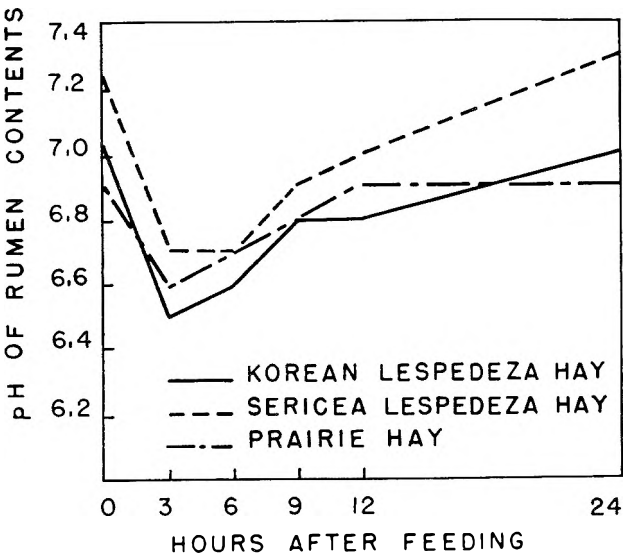

Fig. 1 The pH of the rumen contents at various hours after feeding.

Between three and 6 hours following feeding the trend of the pH of the rumen is toward an alkaline condition. This trend may be due to a very copious flow of saliva having an alkaline reaction which passes from the mouth into the rumen (Colin, 1886; McDougall, '48; Markoff, '13; Zuntz, '13). The second possibility is the influence of the mineral content of the ingesta (Ammerman and Thomas, '52).

Upon examination of the data in table 1, one observes that the pattern of pH follows rather closely the per cent of ash in the rumen ingesta. One observes further that the pattern

of pH does not appear to have a close relationship to either the fat percentage or the per cent of fatty acids in the rumen, except during the period between zero and three hours. In view of these trends, it was deemed advisable to make a statistical analysis of the data to determine the magnitude of the apparent relationship existing between the pH of the rumen and these constituents.

The correlation coefficients indicating the relationship between certain chemical constituents of the rumen ingesta and the pH of the rumen ingesta are presented in table 2 and figure 2. A very low correlation exists between the percentage of fat and the pH of the rumen contents. A negative correlation was observed in the case of the fat content of Sericea lespedeza and prairie hays and the rumen pH, while a positive correlation was found in the case of Korean lespedeza hay. When the data for all hays were combined, a negative correlation existed. None of these correlations was significant, however, and the positive variation in the case of the Korean lespedeza hay may well have been due to chance. However, when one examines the fat percentage of the Korean lespedeza in table 1, it is observed that this value is abnormally high for some unknown reason, but it may have had its origin in the small amount of foreign material present in the hay.

A positive, though insignificant, correlation was observed betwen rumen pH and the per cent of fatty acids in the rumen ingesta, which would indicate that the influence of the fatty acids per se on pH is at least partially neutralized, since they normally give an acid reaction when in solution. Thus it appears that strong chemical forces other than the fatty acids are present in the rumen. The chemical forces involved appear to be those shown by the buffering effects of the saliva and of the ingested material. The correlation between fatty acids and pH was highest in the trial in which prairie hay was fed. It is interesting to note that this is the sole instance in which there was not a highly significant relationship be-

TABLE 2

Simple correlation coefficients between pH of rumen contents and certain chemical components of the rumen ingesta

SUBJECT	PER CENT FAT	PER CENT FATTY ACIDS	PER CENT ASH
		Korean lespedeza hay	
pH	+ 0.31	+ 0.12	+ 0.92 [1]
		Sericea lespedeza hay	
pH	— 0.16	+ 0.08	+ 0.92 [1]
		Prairie hay	
pH	— 0.58	+ 0.50	+ 0.73
		All hays	
pH	— 0.09	+ 0.15	+ 0.89 [1]

[1] Significant, $p = < 0.01$.

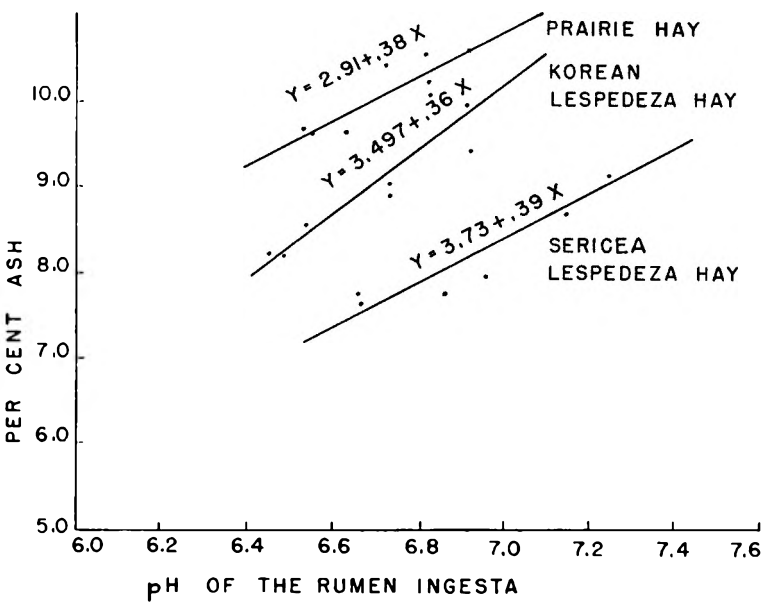

Fig. 2 The relation of the pH of the rumen ingesta to the per cent ash in hays fed to fistulated steers.

tween the ash content of the ingesta and rumen pH, though this relationship did approach significance.

A high degree of relationship was found to exist between the ash content of the ingesta and the pH of the rumen. All correlations within a given type of hay were significant, with the exception of prairie hay, previously mentioned. These findings are in accord with the recent report of Ammerman and Thomas ('52) on the apparent buffering capacity of forage juices. It appears that the ash, moving out of the rumen more slowly than the other constituents of the ingesta, exerts a buffering action and should be considered as a factor influencing rumen pH.

SUMMARY AND CONCLUSIONS

A study has been made to determine the apparent correlation between the pH of the rumen and the fat, fatty acid and ash content of the ingesta from Korean lespedeza, Sericea lespedeza, and prairie hays at given intervals during rumen digestion.

A significant positive correlation was found to exist between the ash content of the ingesta and the pH of the rumen. This relationship appears to be due to the buffering capacity of the ash which, together with the buffering action of the saliva, tends to keep the pH of the rumen contents within rather narrow limits.

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RELATION OF PROTEIN AND FAT INTAKE TO GROWTH AND CORNEAL VASCULARIZATION IN GALACTOFLAVIN-PRODUCED ARIBOFLAVINOSIS

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ONE FIGURE

Although the relationship of riboflavin to protein and fat metabolism has been frequently studied, the conclusions drawn have been complicated by the presence in the animal of riboflavin which might have been derived either from tissue stores or from the intestinal flora. The recognition that certain riboflavin analogues are capable of acting as riboflavin antagonists seemed to permit a new approach to various riboflavin problems because of the possibility of producing more advanced stages of riboflavin deficiency.

In this report, therefore, are presented the results of experiments in which the growth, food consumption, survival time and occurrence of vascularization of the cornea of rats on riboflavin-low diets with and without an antagonist have been studied, using various protein and fat levels in the rations supplied. Particularly, studies of the influence of protein-low diets in riboflavin deficiency were inviting because there seemed to be little information in the literature as to the action of such diets on growth and food consumption. Galactoflavin was the riboflavin analogue used because

it had been shown to be a good riboflavin antagonist in animals (Emerson et al., '45; Stoerk and Emerson, '49; Woolley, '52).

METHODS AND MATERIALS

Albino rats of the Sherman strain from a homogeneous colony known to us for 12 years were used. For each series of experiments, 10 to 50 litters, born within three days of each other, were transferred within one to three days after birth to a purified diet containing adequate supplements of all known nutrients. Details as to the composition of and results obtained with this diet have been reported (Kaunitz, '52). At the time of transfer, the young were pooled, the sexes separated, and each mother received the same number of young.

At 22 to 24 days, the young were weaned, ear-marked and weighed; at 28 days, they were reweighed and distributed into matching groups of 5 to 8 young, the average weights of which were identical at 24 and again at 28 days. This procedure reduced the probability that subsequent differences in the average weights of the groups would be due to inherent differences in growth tendencies. At this time the groups were transferred to the experimental diets.

The diets were made up of varying amounts of casein, lard and cerelose, their total comprising 93.5% of the diet. To this were added 4% salt mixture (U.S.P. No. 2), 2% cellulration, 0.5% calcium carbonate and, per kilogram, 1 gm of choline, 1 gm of inositol, 300 mg of para-aminobenzoic acid, 100 mg of nicotinic acid, and 10 mg of vitamin K.[1] The other water-soluble accessory factors were supplied by feeding, 4 times weekly, two drops of a suspension containing, per milliliter, 4 mg of thiamine chloride, 8 mg of riboflavin, 8 mg of pyridoxine, 20 mg of calcium pantothenate, 5 mg of folic acid, 50 μg of biotin, 10 μg of vitamin B_{12} and 50 mg of ascorbic acid. The fat-soluble vitamins were administered by feeding three drops weekly of a linoleic acid suspension containing,

[1] Synkayvite.

per milliliter, 10 mg of free alpha-tocopherol,[2] 50 mg of alpha-tocopherol acetate, 0.5 mg of vitamin D_2 and 5 mg of beta-carotene.[3] Riboflavin was omitted when desired.

For the galactoflavin studies,[4] the aqueous suspension contained 60 mg of this substance, 32 mg of riboflavin, and the other supplements as above. Again riboflavin was omitted when necessary. Of these suspensions, two or three drops were fed 6 times weekly, allowing a daily intake of 3 to 5 mg of galactoflavin and 1.5 to 2.5 mg of riboflavin (for the controls).

In the earlier part of the studies, alcohol-washed casein was used; however, it had been found to contain 1 μg of riboflavin per gram (Cannon et al., '45), a value which we confirmed by microbiological assay.[5] Considering that this could allow a daily intake of 3 μg of riboflavin to rats on diets with 30% casein, further purification seemed desirable. This was done by thoroughly mixing 10 kg of the casein with 2.5 kg of oxidized lard, permitting this mixture to stand at room temperature for one week, and thoroughly extracting the fat by repeated alcohol and petroleum ether treatment. When dry, the casein was washed with dilute acid according to the method of Cannon et al. ('45). The riboflavin content of this sample was below the limit of accurate microbiological determination. In feeding experiments with riboflavin-deficient diets, the extracted casein produced a more pronounced depression of growth and appetite than the alcohol-washed material; but,

[2] We are greatly indebted to Dr. Leo A. Pirk of Hoffmann-LaRoche, Inc., Nutley, N. J., who has supplied us with most of the synthetic vitamins used and has on many occasions given us very valuable advice.

[3] Vitamin D_2 and crystalline beta-carotene were received through the courtesy of the Sterling-Winthrop Research Institute, Rensselaer, N. Y., and the Barnett Laboratories, Long Beach, Calif., respectively.

[4] We are indebted to Dr. Herbert C. Stoerk of the Merck Institute for Therapeutic Research, Rahway, N. J., for providing us with galactoflavin and for advising us as to many of its properties. Merck and Co., Rahway, N. J., also furnished us with vitamin B_{12}.

[5] The microbiological assays for riboflavin were carried out according to the U.S.P. XIV method by the Laboratory of Industrial Hygiene, New York City.

with the addition of riboflavin, the two samples led to identical growth. Thus it appeared that most of the riboflavin had been removed, while the biological value of the treated casein had not been impaired.

The examinations of the eyes were carried out by means of a Zeiss slit-lamp after dilation of the pupils with 5% homatropine. As a measure of the severity and progress of the condition, the capillary loops present in one corneal quadrant and their distance from the center of the cornea were recorded.

Growth and food consumption were measured in the usual way. The body weights were recorded by plotting the logarithm of the weight in grams against the reciprocal value of the age (Zucker and Zucker, '42).

EXPERIMENTAL

In growth studies (table 1) using complete diets containing 20% lard and 5 to 74% casein, the resulting weight increases were roughly parallel to the dietary protein levels up to the 30% level. With 74% casein, the weights were lower than those with 30%, being similar to those obtained with 18%. On 5%, the young usually lost a few grams during the first two weeks and then grew slowly.

The daily caloric intake increased for the rats on 18 and 30% casein from about 35 to about 50 cal. during the first 4 to 6 weeks on the diets. With 74% protein, the average intake was 15 to 20% lower. On 5% protein, the food intake was about half that of the animals receiving 30% and remained rather constant.

With riboflavin-deficient diets containing 18 and 30% casein (table 1), the average food consumptions for the first 5 weeks were about two-thirds, and the over-all weight increases for this period, one-third to one-half those of the controls; thereafter the weights became constant, confirming earlier observations (Sure, '41; Mannering and Elvehjem, '44).

With 74% casein, the food consumption of the deficient animals was nearly the same as with 18%, but the weight gain

of the high-protein group was significantly greater. On 5% protein, no differences in growth and food consumption between the high- and low-riboflavin groups were observed, even after 4 months.

When 3 to 5 mg of galactoflavin were fed daily to the animals on the riboflavin-deficient diets, the appearance of the rats after one or two weeks was comparable to that of rats kept on identical diets without galactoflavin after several

TABLE 1

Results of growth studies[1]

SEX	CASEIN	WEIGHT INCREASE	DAILY CALORIC INTAKE
	%	*gm*	
♂	5	8 (7)	28 (28)
♂	18	50 (130)	35 (..)
♂	30	68 (155)	37 (55)
♀	18	31 (105)	26 (41)
♀	30	52 (96)	.. (39)
♀	74	47 (104)	22 (35)

[1] Average weight increase and daily food consumption for 5 weeks after rats had been placed at 28 days on purified diets with high and low riboflavin supplements, various casein levels, and 20% lard. The results with the riboflavin-high groups are given in parentheses. Totals of 188 animals on the complete and 122 on the deficient diets were used for the calculation of averages.

months. The development of allopecia, dermatitis, blood-caked whiskers and so forth was the same for protein levels of 5 and 30%, but these signs appeared earlier among animals on 74%.

The results as to growth and food intake with 5 and 30% casein are shown in figure 1; the rest of the galactoflavin data are summarized in table 2. Regardless of the protein level, most of the animals on the deficient diets started losing weight within a few days. The weight curves of the groups on 5 and 30% did not differ sharply from each other in two experiments; in one, the weights of those on 30% were lower than those of the rats on 5%. The group of 8 rats on

TABLE 2

Results of galactoflavin administration[1]

GROUP	NUMBER AND SEX	CASEIN	FAT	AVERAGE BODY WEIGHT Age (days) 28	35	42	49	56	63	AVERAGE DAILY CALORIC INTAKE Weeks on diet 1	2	3	4	Ave. for whole period
		%	%	gm										
H	8 ♀	5	20	57	51	45	40	40	37	22.8	17.6	12.4	10.5	17.6
I	8 ♂	5	20	66	66	61	58	51	50	24.3	19.1	13.3	10.0	16.7
G	5 ♀	5	20	59	62	61	54							
										30.6	26.7	17.6		25.2
G[2]	5 ♀	5	20	59	62	62	59							
I	8 ♂	10	20	66	69	62	58	53	50	25.2	18.6	14.8	11.9	17.6
H	8 ♀	30	20	57	59	55	48	44	40	19.1	14.3	11.9	10.9	18.1
I	8 ♂	30	20	66	76	72	68	60	55	22.9	15.7	15.2	11.9	16.2
G	5 ♀	30	20	59	64	55	44							
										20.0	13.3	17.6		17.2
G[2]	5 ♀	30	20	59	70	61	60							
I	8 ♂	74	20	66	69	61	55	47	41	21.0				
H	6 ♂	5	0	62	51	44	42	39	35	15.8	15.0	12.8	11.6	13.9
H	6 ♂	30	0	62	57	55	53	50	48	15.0	16.1	13.9	12.4	14.3

[1] Influence of daily administration of 3 to 5 mg of galactoflavin to rats placed at 28 days on riboflavin-low diets containing various protein and fat levels. Identical letters indicate matching groups of the same experimental series.

[2] High-riboflavin supplement; paired feeding with the corresponding riboflavin-deficient group of the G series.

74% casein reached weights significantly below those of animals on lower protein levels.

The caloric intakes of these groups did not differ sharply from one another. The rats on 5% protein ate, in one instance, more than those on 30%; but in the other two series the consumptions were identical. Food intakes of the animals on 74% casein could be measured only during the first

Fig. 1 Influence of galactoflavin on average weights and food intakes of groups of 8 female rats on purified diets containing different protein and riboflavin levels.

Curve 1: 30% casein, riboflavin-high plus galactoflavin
Curve 2: 30% casein, riboflavin-low plus galactoflavin
Curve 3: 5% casein, riboflavin-high plus galactoflavin
Curve 4: 5% casein, riboflavin-low plus galactoflavin
Curve 5: 5% casein, riboflavin-high, no galactoflavin

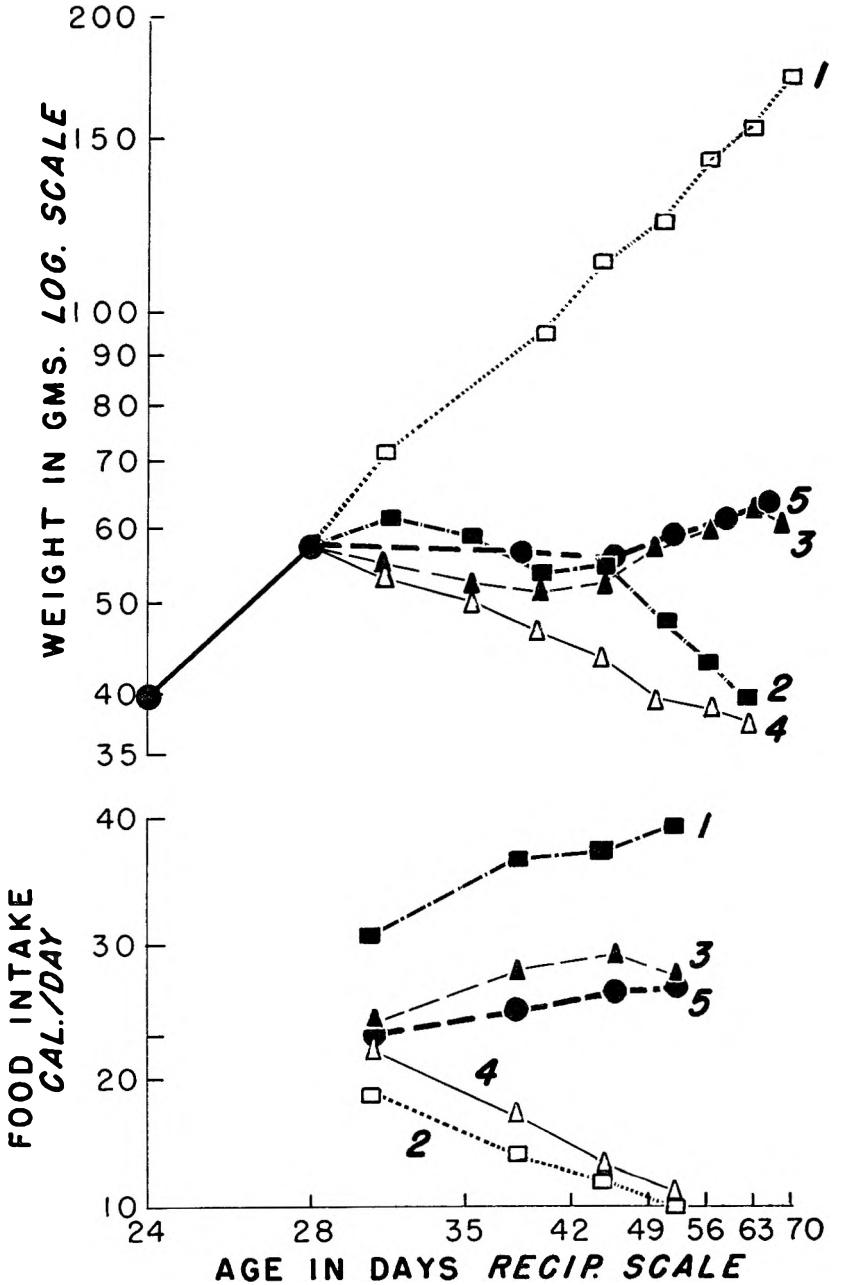
WEIGHT IN GMS. LOG. SCALE
200
150
100
90
80
70
60
50
40
35
1
5
3
2
4
FOOD INTAKE CAL./DAY
40
30
20
10
1
3
5
4
2
24
28
35
42
49
56
63
70
AGE IN DAYS RECIP. SCALE

Figure 1

days of the experiment because of their short survival time, but the data indicated that they ate less than the rats on lower protein levels.

The average survival time of the animals on the 5 or 30% protein diet was only 29 to 37 days (average, 33 days), and there was no difference between the groups. Those on 74% protein died earlier.

In figure 1 are also shown the results of experiments in which the animals were supplemented with galactoflavin and riboflavin in a ratio of approximately 2:1. These control experiments were necessary in order to determine whether, under the experimental conditions, galactoflavin was toxic or acted as a true riboflavin antagonist as it had in earlier studies (Emerson et al., '45). When the rats were fed 5% casein, their weight and food consumption were identical with those of the corresponding rats without galactoflavin. On 30% casein, the females receiving both galactoflavin and riboflavin grew at the rate to be expected for normal females according to Zucker and Zucker ('42). Paired feeding experiments (table 2) demonstrated that rats on galactoflavin and riboflavin grew substantially better than their mates receiving no riboflavin. Females kept on galactoflavin and riboflavin reared normal litters.

It has been repeatedly noted that high-fat diets aggravate the riboflavin deficiency state in rats, but it is not quite clear whether this is due to a true metabolic effect or to decreased bacterial synthesis (Mannering et al., '41; Shaw and Phillips, '41; Elvehjem, '46; Czaczkes and Guggenheim, '46; Forbes et al., '46).

In these studies diets containing 0, 10 and 20% lard and casein levels of 5 to 30% were used. In agreement with the literature, it was observed that the differences in growth and food intakes between the riboflavin-deficient and -sufficient groups increased with the fat content of the diet. When fat-free diets containing 2% linoleic acid and 5% casein were fed, there was again no difference in growth and food consumption between the groups with and without riboflavin.

In table 2 are shown results of experiments with riboflavin-deficient animals on fat-free diets with galactoflavin supplements. The caloric intakes of those on 5 and 30% casein were similar, but the weights and the survival times of the latter were better than those of animals on 5% — which is in contrast to the observations with the high-fat diets.

The corneal capillarization in riboflavin-deficient rats first noted by Bessey and Wohlbach ('39) in animals on 18% casein was found not to be accompanied by changes in the corneal transparency or by alteration of the epithelial cells, at least in the earlier stages of the disease. The histological changes noted in the current studies were in agreement with the above findings. In the first stages, capillary loops from the superficial perilimbal plexus sprouted into the clear cornea, the capillaries lying underneath the corneal epithelium in the superficial layers of the stroma. At this time, no demonstrable signs of edema or infiltration or of epithelial or endothelial changes were noted. Later, the capillaries penetrated into deeper layers of the corneal stroma, but again without histological signs of inflammatory reaction; epithelial edema now occurred quite frequently. These histological changes were observed on all protein and fat levels.

The incidence and the time of onset of the disease encountered under various conditions are shown in tables 3 and 4. Differences between groups were manifested not only by a higher incidence but also by more advanced stages of the disease. With 5% casein and 20% lard, the condition developed equally with high or low riboflavin supplementation. With higher protein levels and high riboflavin intake, the changes never occurred. Increase of the dietary protein significantly delayed the disease in riboflavin deficiency; but, even so, almost all rats were affected after 6 to 7 weeks on the diet, when they were 10 to 11 weeks old. These findings agreed well with the studies of growth and food consumption.

When the deficiency was enhanced by galactoflavin supplements, the occurrence of the corneal disease did not differ significantly from that in deficient animals without galacto-

flavin. With 5% protein, the disease again developed regardless of the riboflavin supplement. Higher protein levels in riboflavin-low diets significantly delayed the onset of the disease, even with galactoflavin supplements. This is in contrast to the findings as to growth and food consumption.

TABLE 3

Incidence of corneal vascularization among rats placed at 28 days on riboflavin-high and riboflavin-low diets containing various protein and fat levels[1]

GROUP AND SEX	CASEIN	FAT	RIBOFLAVIN SUPPLEMENT	INCIDENCE					
				Weeks on diet					
				2	3	4	5	6	7
	%	%							
E ♂	5	20	+				13/18	8/9	
F ♂	5	20	+	3/8		7/7			7/7
I ♂	5	20	+	2/8	6/8	8/8			
E ♂	5	20	0			9/9		9/9	
F ♂	5	20	0		4/8	8/8			8/8
E ♂	18	20	0			2/9		9/9	
F ♂	18	20	0		2/8	7/8		8/8	
E ♂	30	20	0	2/9				8/9	
F ♂	30	20	0		3/8		4/8	7/8	
I ♂	30	20	0		1/8	3/8			7/8
F ♂	5	0	+	2/8		3/8			8/8
I ♂	5	0	+	0/8	2/8	5/8			
F ♂	5	0	0		5/7	8/8			8/8
F ♂	18	0	0		1/7	3/8		6/8	
I ♂	30	0	0		0/8	2/8	5/8		

[1] The denominator denotes the number of animals used; the numerator, those with lesions. Identical letters indicate matching groups of the same experimental series.

The influence of the fat content of the diet was again noticeable in that the deficient groups on fat-free rations receiving galactoflavin developed the disease later than their controls provided with 20% lard.

In continuation of previous studies (Kaunitz et al., '52; Kaunitz, '53), the influence of highly oxidized lard on growth, food consumption and corneal vascularization in the riboflavin deficiency state was studied. The deficiency syndrome was en-

hanced on all protein levels by the presence in the diet of 20% oxidized lard; corneal vascularization occurred much earlier than in rats receiving fresh lard.

TABLE 4

Incidence of corneal vascularization among rats placed at 28 days on riboflavin-high and riboflavin-low diets containing various casein and fat levels and supplemented with 3 to 5 mg of galactoflavin daily [1]

GROUP AND SEX	CASEIN	FAT	RIBOFLAVIN SUPPLEMENT	INCIDENCE			
				Weeks on diet			
				1	2	3	4
	%	%					
G ♀	5	20	+		1/5	4/5	5/5
H ♀	5	20	+	2/4		3/4	4/4
G ♀	5	20	0		0/5	5/5	5/5
H ♀	5	20	0	4/8	6/8	8/8	8/8
I ♂	5	20	0		2/8	5/8	7/8
I ♂	10	20	0		0/8	4/8	6/8
G ♀	30	20	0		0/5	0/5	3/5
H ♀	30	20	0	1/8		3/8	3/8
I ♂	30	20	0			3/8	3/8
I ♂	74	20	0			1/8	1/7
H ♂	5	0	0	2/6	3/6	5/6	4/4
H ♂	30	0	0	0/6		0/6	4/6

[1] The denominator denotes the number of animals examined; the numerator, the number with lesions. Identical letters indicate groups of the same experimental series.

DISCUSSION

The administration of galactoflavin to rats on riboflavin-deficient, purified diets leads to a much more rapid development of the deficiency state than in controls without galactoflavin. Inasmuch as galactoflavin had no toxic effects when administered simultaneously with riboflavin, it can be concluded that the observed changes were due to the action of galactoflavin as a true riboflavin antagonist. The comparatively slow development of the deficiency disease in animals without galactoflavin is most probably due to the presence of some riboflavin in the tissues or to its formation by the intestinal flora; whatever the source of this riboflavin, it is neutralized by galactoflavin.

The studies of growth and food consumption with various protein levels and galactoflavin supplements permitted the conclusion that the utilizations of protein and of riboflavin are mutually limiting. High dietary protein levels cannot be utilized if riboflavin is rigidly restricted, and vice versa.

This mutual limitation between dietary riboflavin and protein is further borne out by the observation that urinary excretion of riboflavin increases steeply on low protein levels (Sarett et al., '42; Czaczkes and Guggenheim, '46) and by the data on the riboflavin content of the tissues of animals on high and low protein levels with and without riboflavin supplements. The riboflavin content of rat carcasses was found to be constant regardless of protein and riboflavin intake (Sarett and Perlzweig, '43). These authors and later examiners (Ferrebee and Weissman, '43; Singher et al., '44; Reisen et al., '46) observed that the riboflavin content of the liver decreased equally with a low riboflavin or a low protein-high riboflavin intake.

Some of the data for the animals on galactoflavin suggested that protein at a high level may even become toxic. This would be similar to the metabolic changes in pyridoxine deficiency (Foy and Cerecedo, '41), and may be relevant to the fact that the catabolic products of tryptophan in riboflavin-deficient rats are different from those of normal animals (Porter et al., '48; Junqueira and Schweigert, '48; Henderson et al., '51). Although the differences which suggest this toxicity are small, they seem to be significant.

With regard to the action of fat, the data may contribute to the discussion of whether the increase of the riboflavin requirement produced by fats is due to a true metabolic effect or to some other mechanism, such as inhibition of riboflavin synthesis by the intestinal flora. The circumstance that a fat-free diet delays the development of the deficiency state in riboflavin-deficient animals provided with galactoflavin suggests that the effect is of a true metabolic nature, if it is permissible to assume that the galactoflavin neutralized most of the animal's riboflavin reserves.

The fact that fat-free, high-protein rations delayed the onset of the corneal changes may indicate that protein utilization in riboflavin deficiency is better in the absence than in the presence of fat. Such a conclusion is also suggested by the results of the growth studies with high- and low-fat diets, because the animals receiving galactoflavin and no fat did better on 30% than on 5% casein, in contrast to those on high-fat diets.

After corneal changes had been described as characteristic of riboflavin deficiency (Bessey and Wohlbach, '39), it was pointed out (Sydenstricker et al., '46) that similar changes could be produced by protein deficiency. Various authors eventually formed the opinion that the condition is rather unspecific and might occur as a consequence of the deficiency of any essential nutrient.

Critical evaluation of the published reports makes the latter generalization questionable. One finds that, in those studies in which corneal vascularization had been observed despite the presence of large riboflavin supplements, the protein accessible to the animals had been purposely or by chance kept at a low level (György, '42; Bietti, '50), or that the intake of one of the essential amino acids had been restricted (Albanese and Buschke, '42; Sydenstricker et al., '47), which is tantamount to protein deficiency (Cannon, '45), or that the disease occurred at late stages of other deficiencies when, again, the protein intake of the animals must have been low. We have found no evidence in the literature that the disease may occur at a relatively high protein intake with sufficient riboflavin supplements.

The fact that corneal disease develops among rats on protein-low diets regardless of the riboflavin supplement could suggest that protein deficiency is the cause of the disease. However, raising the protein level of the diet only delays the onset of the disease in riboflavin deficiency; it does not prevent it. These observations again lead to the conclusion that riboflavin and protein are mutually limiting factors. Although it is true that the deficiency of any of several fac-

tors may limit protein utilization, the disturbance resulting from riboflavin deficiency is the only one which has been observed to produce the disease on a relatively high protein intake. Until such time, therefore, as the disease can be produced under the same conditions with other deficiencies, the possibility that riboflavin plays a rather specific part in the development of the corneal changes cannot be ruled out.

SUMMARY

The influence of dietary casein levels ranging from 5 to 74% and of fat levels ranging from 0 to 20% upon rats on high and low riboflavin intakes with and without galactoflavin supplementation was studied with regard to growth, food consumption, survival time and corneal vascularization.

On riboflavin-high diets containing 20% lard, weight increases ran roughly parallel to the protein level up to 30%. With 74% casein, the weights were similar to those on 18%. The daily caloric intakes were about 28 cal. for the animals on 5% casein, eventually about 50 for those on 18 or 30%, and about 40 for those on 74%.

On riboflavin-low diets with 18 or 30% casein, food consumption was about two-thirds and growth one-third to one-half those of the controls. With 5% protein, there was no difference in growth or food consumption between the animals on high and low riboflavin intakes. With 74% casein, the food consumption was about the same as that of animals on 18%, but those receiving 74% grew better.

Animals on all riboflavin-low diets with 3 to 5 mg of galactoflavin daily rapidly developed the deficiency state. Regardless of the protein level, they lost weight almost immediately; those on 74% lost the most. The daily food intake was about 15 cal.: animals on the highest protein level showed the lowest consumption. The survival time of all groups was only about 30 days; those on 74% protein died first.

With simultaneous galactoflavin and riboflavin supplements, no toxic effects on growth, food consumption or fertility were noted.

It was concluded that riboflavin and protein are mutually limiting factors and that, at a high level, protein may become toxic in riboflavin deficiency.

High dietary fat levels accentuated the deficiency state, the galactoflavin data suggesting that this effect is a true metabolic one and that protein utilization in riboflavin deficiency is probably better in the absence than in the presence of fat.

Corneal vascularization occurred equally in all animals on 5% casein, regardless of riboflavin or galactoflavin intake. Higher protein levels delayed the development of the condition. On fat-free diets its onset was further delayed. The conclusion was drawn that the possibility cannot be ruled out that riboflavin deficiency plays a more specific part in this type of corneal vascularization than is often believed.

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THE DETERMINATION OF METABOLIC FECAL NITROGEN

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FOUR FIGURES

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The metabolic fecal products originate in the body, either in the course of its endogenous catabolism or of the digestive processes themselves (Schneider, '35). Under conditions of adequate, supermaintenance feeding, the digestive processes are evidently the dominant sources of the nitrogenous constituents of the metabolic fecal products. Conceivably, these products contain considerable amounts of unabsorbed residues of bile, pancreatic juice, gastric juice and the intestinal juices.

The intestinal mucosa also contributes to the metabolic products of the feces. This phase of the subject has been studied histologically by Leblond and Stevens ('48) in a most interesting report on the constant renewal of the intestinal epithelium in the albino rat. They demonstrated a constant migration of mucosal cells from the crypts of Lieberkühn along the surfaces of the villi to an "extrusion zone" at the tips of the villi, from which they were ejected into the lumen of the intestine. From measurements of the duration of mitosis in the crypts and the number of mitoses at any one time, it was estimated that epithelial cells lived an average of 1.57 days in the duodenum and 1.35 days in the ileum. Extrusion zones at the tips of intestinal villi have been found in man, cat, rabbit and mouse after treatment with various fixatives.

In the large intestines the metabolic fecal products, whatever their origin, serve as nutrient media for the resident bacteria, which may make up a predominant part of the fecal nitrogen and fecal dry matter on low-residue diets (MacNeal, Latzer and Kerr, '09; Osborne and Mendel, '14).

Measurement of the metabolic fecal nitrogen is important in the determination (1) of the true digestibility of dietary protein in experiments in which an accurate knowledge of the wastages of dietary nitrogen both in digestion and in metabolism is essential, and (2) of the nitrogen (protein) requirements of man and animals. The metabolic fecal nitrogen, representing wastages of body nitrogen that must be replaced to maintain the nitrogenous integrity of the body, was recognized as an integral part of the protein requirement of the body by Tsuboi (1897), by Thomas ('09), and by Daly and Mirsky ('52). The observations of Tarver and Schmidt ('42), of Friedberg ('47) and of Wheeler et al. ('49), using dietary amino acids labeled with radioactive isotopes of sulfur or of carbon, demonstrate the replacement syntheses in the intestinal mucosa and the pancreas.

The amount and nature of the metabolic fecal products have been studied from early times by collecting and analyzing the feces of men and animals subsisting upon diets containing minimum amounts of nitrogen (Parkes, 1867; Rubner, 1879; Rieder, 1884; Prausnitz, 1897). The relationship of the metabolic fecal nitrogen so determined to the intake of dry matter has been found to be a close one when the intake of food is sufficient for maintenance of body weight and the fiber content of the diet is low (Mitchell, '24; Schneider, '34; Chick et al., '35), regardless of the proportion of fat and carbohydrate in the diet (Mitchell, '34). The incorporation of fiber in the diet increases the proportion of metabolic fecal nitrogen to dry matter consumed (Mitchell, '24).

Since the incorporation in a nitrogen-free diet of lactalbumin (Mitchell, '24), whole egg and pork (Mitchell and Carman, '24) in the case of rats, meat in the case of dogs (Mendel and Fine, '12), milk proteins in pigs (Schiftan, '32),

and egg albumin and wheat gluten in human subjects (Hawley et al., '48) did not appreciably increase (or decrease) the fecal output of nitrogen, it may reasonably be inferred that the inclusion of protein in the diet does not disturb the ratio of metabolic fecal nitrogen to the dry matter consumed. Hence, the ratio determined on a protein-free diet should apply to experimental periods involving the feeding of protein-containing diets. Such evidence is the essential basis for the thesis that the excretion of metabolic fecal nitrogen is unaffected by the inclusion of protein in the diet of man and animals. Using an entirely different approach to the problem, Lofgreen and Kleiber ('53) obtained results with calves, varying from two to 10 weeks of age and subsisting on a purified liquid diet, that "fit well with the general trend of other data" secured by the feeding of very low-nitrogen rations, such as the tabulation given by Mitchell ('26, table 6) for monogastric animals on diets low in fiber.

The thesis that the metabolic fecal nitrogen can be measured on a protein-free diet was attacked by Titus ('27) in experiments on steers receiving diets varying progressively in protein (alfalfa) and in fiber (paper pulp). Titus showed that, within the range of nitrogen intake investigated, there was a linear relationship between the fecal nitrogen, corrected to an 80% water content, and feed nitrogen, but for reasons not at all obvious he refused to accept the intercept of this regression line on the fecal nitrogen axis as the value for metabolic fecal nitrogen. His conclusion "that the amount of nitrogen in the feces of a steer consuming a nitrogen-free ration may not safely be taken as a measure of the amount of metabolic nitrogen resulting from the ingestion of an equal weight of alfalfa, or other feeding stuff," is made without any direct proof, since he did not determine the fecal nitrogen on a nitrogen-free diet. This phase of his experiments would not merit reference here except for the fact that the conclusion cited is referred to frequently in recent literature, generally under the misconception that Titus advocated the determination of metabolic fecal nitrogen by

extrapolation of the regression line of fecal nitrogen on nitrogen intake, rather than by direct measurement on a nitrogen-free diet (for example, Bell et al., '50).

Bosshardt and Barnes ('46) demonstrated with mice a linear relationship between the fecal nitrogen per unit of food consumed and the level of dietary protein in the diet, except for very low levels of nitrogen intake, for which low values for the fecal-nitrogen-to-food ratio were obtained. The aberrant ratio with mice on diets restricted to 30% of the normal caloric requirement, obtained on nitrogen-free feeding, was taken to indicate that "metabolic fecal nitrogen values determined with protein-free or low-protein diets are not safe indices of the metabolic fecal nitrogen under conditions of protein feeding." The similarly aberrant ratio obtained at a dietary nitrogen concentration of 0.287%, in another group of mice fed ad libitum on different levels of dietary nitrogen, is discarded in plotting the regression line. Apparently, the mouse exhibits a decline in the ratio of fecal nitrogen to dry matter consumed when subsisting on a very low-nitrogen diet for a short period of time, an eventuality that occurs with the rat only after protracted protein inanition, according to Seegers ('38).

In this confusing situation with reference to the measurement of metabolic fecal nitrogen, further investigation specifically designed to test the validity of direct measurement by nitrogen-free feeding seemed advisable. The results secured experimentally and a survey of similar tests from other laboratories establish beyond reasonable doubt that the direct measurement is valid and that the indirect method of extrapolating a regression line of fecal nitrogen on nitrogen intake is consistent with the direct method, since the regression is rectilinear to the intercept on the fecal nitrogen axis.

EXPERIMENTAL PROCEDURE

Seven experiments on growing rats were undertaken to determine the biological value of whole egg protein at different levels in comparable diets. The protein levels tested

were approximately 0, 4, 8, 12, 16 and 20%. In the course of these studies the ratio of fecal nitrogen to air-dried food consumed was measured at each protein level. The data with reference to urinary nitrogen and biological value of the protein will be reported later.

Each experiment contained 8, 10 or 12 rats, paired according to sex, initial weight (ranging from 50 to 70 gm) and, generally, litter membership. The 4, 5 or 6 pairs of rats were fed equal amounts of food within pairs throughout a 4-period test, generally; each period consisted of a 7-day pre-feeding and a 7-day collection period. A typical experimental plan was the following: period 1, 4% whole egg protein; period 2, 16% vs. 20% whole egg protein; period 3, 20% vs. 16% whole egg protein; and period 4, 4% whole egg protein. Each rat in such a test received the 4% protein ration in periods 1 and 4, the 16% protein ration in either period 2 or 3, and the 20% protein ration in either period 3 or 2. In one experiment, 5 pairs of rats were fed the 4% protein ration in either period 1 or 2, and a nitrogen-free ration in either period 2 or 1.

The number of nitrogen balances obtained at each protein level were: 10 on the nitrogen-free diet, 120 on the 4% protein diet, 40 on the 8% diet, 30 each on the 12% and on the 16% diets, and 10 on the 20% diet. The 4% egg protein was fed in each of the 7 experiments as a reference diet.

The whole egg protein used in all diets was prepared in the laboratory from fresh non-fertile eggs, which were heated a few minutes in boiling water to coagulate the protein, ground and dried at a temperature of about 50°C., defatted with petroleum ether, and ground again after removal of the solvent at a low temperature.

All rations contained 5% of Wesson salts ('32), 1% of sodium chloride, 4% of cellu flour,[1] 1.5% of cod liver oil, and 0.5% of wheat germ oil (2% cod liver oil and no wheat germ oil in one test), 8% of filtered butterfat, lard to make 20%

[1] A product of the Chicago Dietetic Supply House, containing 0.003% nitrogen and 53% crude fiber.

of fat (10% fat in one test, inadvertently), the desired amount of dried defatted whole egg, and enough sucrose and starch to make up to 100%.

The feces and urine were collected separately in individual dishes, and diets, feces and urine were analyzed separately for nitrogen by the Kjeldahl method, using mercury as a catalyst. The heats of combustion of all diets were determined in a bomb calorimeter. The feeding tests were conducted in a temperature-controlled room maintained at 76° to 78°F.

EXPERIMENTAL RESULTS

The results that will be considered in this paper relate to the relationship of the fecal nitrogen, in grams per 100 gm of air-dried feed consumed, to the percentage of protein in the air-dried diets. The relationship is shown graphically for all experiments in figure 1. Each point in the figure is an average of 8 to 24 determinations. A straight line was fitted to the 19 averages by the method of least squares, giving the regression equation

$$Y = 1.3191 + 0.08573X, \quad (1)^{2}$$

in which Y is the ratio of fecal nitrogen to dry matter consumed and X is the percentage of protein (whole egg) in the diet. In this fitting operation, the 19 means available were weighted in accordance with the reciprocal of the variance of the mean per group. The regression coefficient is highly significant ($P < 0.001$), while no significant improvement in fit was secured with a quadratic equation ($P > 0.10$).

For the purpose of determining whether the average ratio secured at a dietary protein level of 0.26% (as low as could be obtained), i.e., 1.436, was homogeneous with the other means at higher protein levels, a straight line was fitted to all means except the one under test, with the following result:

$$Y = 1.3099 + 0.08670X \quad (2)$$

[2] The true digestibility of the egg protein nitrogen computed from this equation is 94.6%. In other experiments we have reported digestibilities of 100% (Bricker and Mitchell, '47) and of 97.5% (Mitchell and Beadles, '50). Such differences as these may result from different temperatures of drying, different degrees of extraction of lipide nitrogen, and possibly other causes.

Solving for Y when X = 0.26 gives a value of 1.332 with a variance of 0.002335. The 10 determinations at X = 0.26 showed a variance of 0.004973. Using the "t" test to measure the significance of the difference between 1.436 and 1.332 gives a probability between 0.2 and 0.3. It may therefore be concluded that the observed ratio at X = 0.26 and the ratios secured at higher values of X were homogeneous. In other

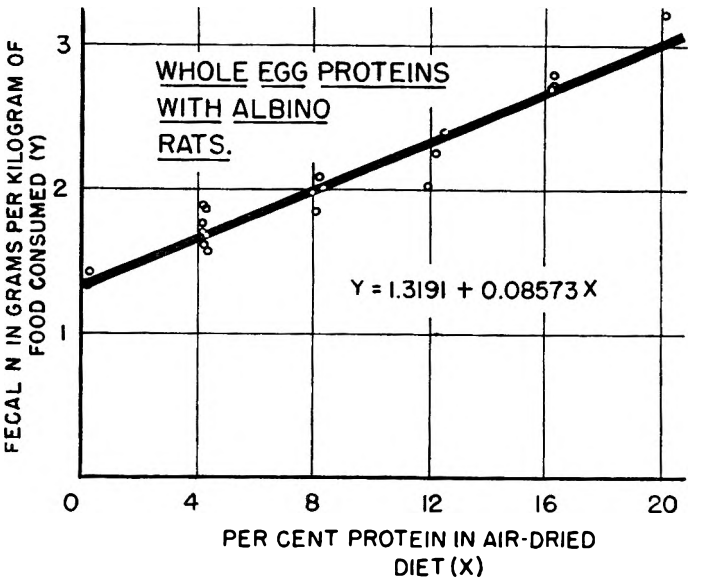

Fig. 1 The regression of the ratio of fecal nitrogen on dry matter consumed on protein content of diet for growing albino rats. Each point is the average of 8, 10, 12, 16, 20 or 24 determinations.

words, the mean ratio of *metabolic* fecal nitrogen to air-dried food consumed, determined in 10 measurements with a diet very nearly free of nitrogen, is indistinguishable statistically from that determined by extrapolation of a regression line based upon 240 measurements of the ratio of *total* fecal nitrogen to dry matter consumed at 5 higher levels of protein intake. Certainly there is no evidence from these observations that under conditions of severe protein restriction or the ingestion of a protein-free diet the intestinal secretion

of nitrogen is markedly decreased, as Bosshardt and Barnes ('46) have reported for the mouse.

The work involved in measuring directly the metabolic fecal nitrogen per unit of dry food consumed, either with a nitrogen-free or with a low egg-protein diet (Mitchell and Carman, '26), is obviously less than that involved in measuring it by extrapolation of a regression line. To determine the slope of this line two points at least must be located, or three points if the linearity of the regression is to be established. If 10 animals are used per group, the direct determination will involve one measurement on each of 10 animals, the indirect determination one measurement on each of 20 or 30 animals. In such a situation, it is important to determine whether the direct measurement is any more or less accurate than the indirect.

The problem is one of comparing the reliability (or precision) of a sample mean with that of an estimated point obtained from a straight line regression equation. As stated above, such an equation was fitted to 250 observations. The fitting was done after establishing that the variance of the arrays of observations of Y (ratio of total fecal N to dry matter consumed) were not significantly correlated ($P > 0.05$) with X (protein content of the diets used). The fact that variances of the observed arrays of Y were not homogeneous was taken into account in the tests of significance involving this regression equation, and gives full validity to such tests.

Let us consider an experiment in which the ratio of metabolic fecal nitrogen to dry food consumed is determined directly on 10 rats on a nitrogen-free (approximately) diet, as compared with an experiment in which this value is determined indirectly by extrapolation of a regression line determined by 10 measurements of the ratio of fecal nitrogen to dry matter consumed (Y) at three levels of any dietary protein (X). We will assume that the observations Y are distributed normally about their respective group means, that the variances of these groups are homogeneous (i.e.,

nearly equal and therefore independent of X), and that the regression of Y on X is a straight line. In the second experiment the protein will be tested at the dietary levels of 4, 7 and 10%. In this experiment the variance of any estimate of Y is expressed, according to Fisher ('44, section 26), as

$$V(Y) = s^2 \left[\frac{1}{n'} + \frac{(X - \bar{X})^2}{S(X - \bar{X})^2}\right] \quad (3)$$

where s^2 is the observed error variance based on n′ observations; n′ is the number of observations, 30; X is the percentage of protein in the diet, and $\bar{X}$ is the mean protein percentage, 7. S stands for summation over the whole sample. Replacing V (Y) by $\frac{s^2}{10}$, the variance of the direct determination of the metabolic fecal nitrogen per unit of dry matter consumed gives a quadratic equation in X with two roots equal to 3.55 and 10.45. This means that, within the interval bounded by these two values of X, the variance of Y estimated from the regression equation is less than that of Y determined directly, while outside this interval the reverse is true. The variance of Y determined at $X = 0$, or very near 0, is less than one-third of the variance of the estimated Y, taken as the intercept of the regression line and the Y axis.

DISCUSSION

The experiments just described demonstrate that for the growing albino rat a linear relationship may be expected between the ratio of fecal nitrogen to dry matter consumed and the level of dietary protein, within a range of from 0 to 20%. Such a result was not obtained for the mouse by Bosshardt and Barnes ('46). It is of interest and importance to investigate the situation with reference to other species of animals.

In the course of his experiments on the biological value for pigs of the proteins in a number of feeds, Schiftan ('32) determined the fecal nitrogen on at least two levels of dietary protein and also the fecal nitrogen on a protein-free diet. Four growing pigs were used in the experiments. The rela-

tionship of fecal nitrogen per 100 gm of dry matter consumed to the protein content of the ration for 4 of the feeds studied is shown in figure 2. The results for potato protein (N × 6.25) were too variable to warrant averaging. Each point in the figure is a mean for at least two pigs. The mean value for

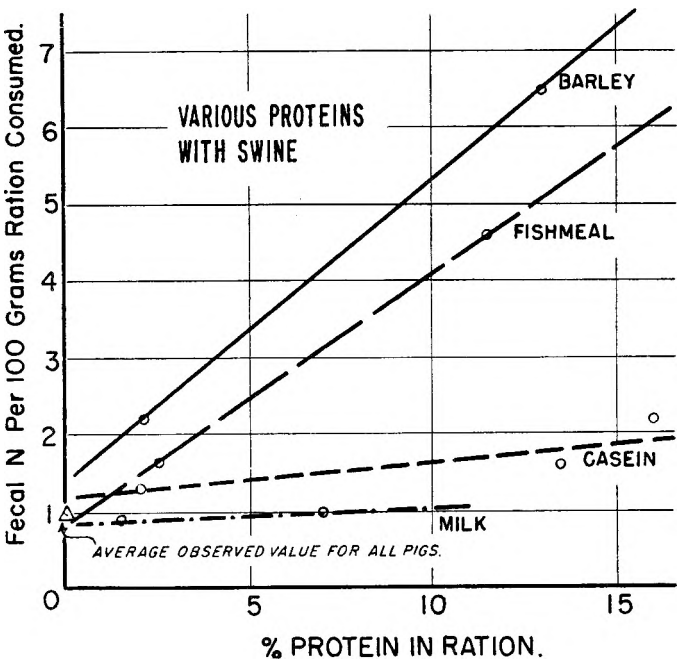

Fig. 2 A graphic presentation of the results of Schiftan ('32) on growing pigs with reference to the relationship of the ratio of fecal nitrogen to dry matter consumed and the protein content of the diets fed. The lines, although drawn with no reference to the results secured on a nitrogen-free diet, clearly converge upon the mean of such results.

the protein-free ration is not considered in drawing the lines through the two or three points secured with each feed, but it is evident that all lines converge upon this value as nearly as the variability of the individual measurements would permit. A confirmation of the complete linearity of this relationship for the pig using soybean protein will be published elsewhere from this Division.

Figure 3 is a reproduction of an unpublished chart furnished by Dr. James B. Allison and published here with his kind permission. It depicts the relationship under consideration based upon observations secured with three dogs during nitrogen balance studies with a soybean protein preparation

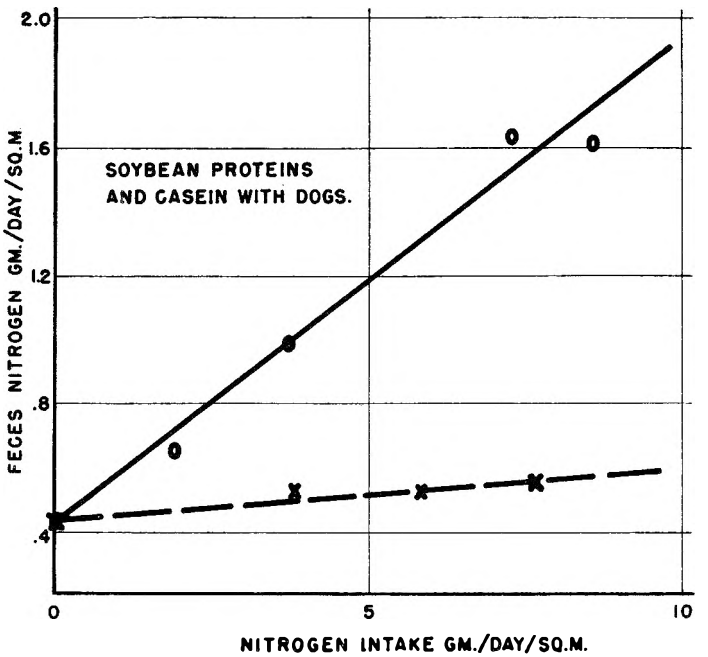

Fig. 3 Average data obtained while feeding three dogs a denatured protein from soybean (O) and a sample of casein (X). Reproduced through the courtesy of Dr. James B. Allison. In comparing this figure with the others, it seems justifiable to assume that the dry matter intake would be in direct proportion to the surface area of animals in confinement.

and casein. Evidently for the dog, as well as for the rat and the pig, the linear relationship between fecal nitrogen and nitrogen intake extends down to the ordinate representing complete protein inanition.

Figure 4 is based upon observations on 8 mature ewes reported by Harris and Mitchell ('41) and secured with various levels of urea added to a low-nitrogen ration con-

taining wheat straw to supply the necessary bulk. The regression line was fitted to the data, exclusive of those secured at about 0.136% nitrogen in the ration, by the method of least squares. The mean observed value of the ratio of fecal nitrogen per 100 gm of dry matter consumed, at the lowest level of dietary nitrogen, with its standard error, was 0.5524 $\pm$ 0.0137; that computed from the regression equation at

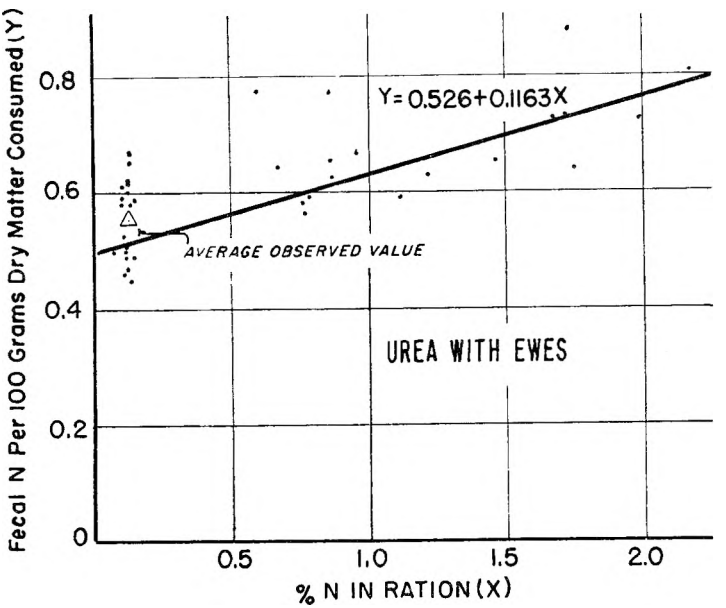

Fig. 4 The relationship between fecal nitrogen (in grams) per 100 gm of dry matter consumed and the per cent nitrogen in the ration for 8 mature sheep receiving various levels of urea incorporated into basal low-nitrogen rations. The data were taken from Harris and Mitchell ('41).

$X = 0.1359$ was 0.5418 ± 0.0403. The difference between observed and calculated values was statistically insignificant with $P > 0.7$. With these data also the linearity of regression extends definitely to $X = 0.1359\%$ dietary N and presumably to the Y ordinate.

CONCLUSIONS

From a series of experiments involving 70 growing rats and 250 nitrogen balance studies in which dried defatted

whole egg at different dietary levels served as the dominant source of nitrogen, it was shown that the ratio of fecal nitrogen to air-dried food consumed is linearly related to the protein content of the diet within a range from 0.26 to 20%. Since the ratio at the lowest dietary protein level, determined directly, is not statistically different from that estimated from the regression equation of the one variable on the other, it seems clear that direct determination of the metabolic fecal nitrogen per unit of dry food consumed is, for the growing rat, a valid method, published results with mice and cattle to the contrary notwithstanding.

The direct measurement of metabolic fecal nitrogen in the determination of the biological value of a protein is more economical of time and of animals than the method involving the establishment of a regression equation between fecal nitrogen and nitrogen intake. It may also be subject to less random error if the regression line is anchored only at relatively high levels of nitrogen intake.

Confirmation of the validity of the measurement of metabolic fecal nitrogen directly by the feeding of a protein-free diet is cited from published experiments on pigs, dogs and sheep.

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