ACHROMATOSIS IN THE FEATHERS OF CHICKS FED LYSINE-DEFICIENT DIETS [1]

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There are several reports in the literature that Broad Breasted Bronze poults fed lysine-deficient diets show a reduced pigmentation of the flight feathers (Fritz, Hooper, Halpin and Moore, '46; German, Schweigert, Sherwood and James, '49; Slinger, Hill, Gartley and Branion, '49; Kratzer, Williams and Marshall, '50; Gartley, Slinger and Hill, '50; Slinger, Gartley and Hill, '50).

Two reports suggest that a relationship between lysine deficiency and pigmentation also exists for certain breeds of chicks. Patrick ('53) observed poor pigmentation in the feathers of New Hampshire chicks fed lysine-deficient diets based on sesame seed oil meal, and McConachie, Graham and Branion ('35) found a decreased pigmentation in the feathers of Barred Plymouth Rock chicks fed low-protein diets which, on the basis of present knowledge, were probably low in lysine relative to other essential amino acids.

Achromatosis of feathers resulting from the consumption of lysine-deficient diets appears to be one of a few examples of a specific symptom caused by an inadequate intake of a single essential amino acid. Consequently, it is a problem of considerable interest as to how the level of dietary lysine affects feather pigmentation.

[1] The data presented in this paper are taken in part from a thesis presented by G. J. Klain in partial fulfillment of the requirements for the degree of Master of Science in Agriculture, University of Toronto, 1956.

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The experiments described herein represent a preliminary approach to the problem. Four breeds of chicks were investigated and found to be susceptible to achromatosis of the feathers when fed a lysine-deficient diet. The amino acid and copper contents of normal and achromatous feathers were compared and attempts made to correct the achromatosis by the addition to the diet of several supplements known to be concerned with feather or hair pigmentation. In addition, a comparison has been made of the tyrosinase activity in poorly pigmented feathers with that in apparently normal feathers. Charles and Rawles ('40) have demonstrated tyrosinase activity in the feather germs of dark feathered chickens and Kratzer and Vohra ('56) found that feather pulp from poults deficient in lysine contained very little tyrosinase activity.

EXPERIMENTAL

The lysine-deficient basal diet was based on expeller processed sunflower seed oil meal. It consisted of 50% ground wheat, 8.56% ground yellow corn, 34.19% sunflower seed oil meal, 1.0% soybean oil, 2.0 dehydrated grass, 2.0% dried buttermilk, 0.75% ground limestone, 1.25% steamed bone meal, 0.25% iodized salt, with the following per 100 pounds of diet: 14.0 gm $MnSO_4$ (technical grade), 0.39 gm riboflavin, 197.0 gm vitamin B_{12}-penicillin supplement (containing 3.0 mg vitamin B_{12} and 2.0 gm procaine penicillin per pound), 3.09 gm dry concentrate of vitamin D_3 (16,500 I.C.U. per gm), 1.53 gm concentrate of vitamin A (250,000 I.U. per gm). The protein content ($N \times 6.25$) of this diet was approximately 24.0% and the lysine content by microbiological assay was 0.65%. When supplemental lysine was used it was added in the form of a commercial preparation containing 95% of L-lysine monohydrochloride.

All feeding experiments were started with day-old chicks and were of 4 weeks duration. Birds were housed in battery brooders and feed and water were supplied ad libitum.

Amino acids were assayed microbiologically using the procedure and medium developed by Henderson and Snell ('48)

with minor modifications. Tween 80 [3] was added to the medium at a level of 0.005% and potassium salts were used in place of sodium salts wherever possible. In the assay for glycine and glutamic acid the medium was adjusted initially to pH 6.0 and in the latter assay asparagine was used in the medium in place of aspartic acid.

The copper content of the basal diet and feathers was determined by the method of Cheng and Bray ('53).

The tyrosinase activity of the feather tissue was measured by a modification of the procedure described by Charles and Rawles ('40) as follows: birds were killed by dislocating the neck and the primary wing feathers were removed by plucking. Beginning at the tip of the quill, small sections were clipped off, using scissors, until about one-half the length of the quill had been removed. A sufficient number of feathers were used to give 1 gm of quill sections from each bird or in most cases from each wing. The 1 gm sample was ground together with 2.0 ml of M/120 phosphate buffer, pH 6.8, in a glass tissue homogenizer until a homogeneous suspension resulted. The suspension was centrifuged at 3500 r.p.m. for 15 minutes and 0.5 ml of clear or slightly cloudy supernatant liquid was transferred to each of two Klett Summerson tubes graduated at 5 ml. To one tube was added 0.5 ml of a solution containing 360 mg of tyrosine, 40 mg of L-dopa and 10 gm of urethane per 100 ml of M/20 phosphate buffer of pH 6.8. To the other tube, which was to serve for a blank reading, was added 0.4 ml of the tyrosine-dopa-urethane solution in M/20 buffer, similar to the above, but containing a 25% greater concentration of the ingredients, and 0.1 ml of a M/50 KCN solution in M/20 phosphate buffer. Thus the final mixture in each tube totalled 1 ml and contained the same concentrations of tyrosine, dopa and urethane. Tubes were plugged with cotton and incubated for 24 hours at 37°C. in the dark. The incubated mixtures were diluted to 5 ml with distilled water and readings ob-

[3] Polyoxyethylene sorbitan monooleate. Purchased from the Atlas Powder Company Limited, Brantford, Ontario.

tained from a Klett Summerson photoelectric colorimeter. A blue filter was used of transmission range, 400–450 mμ.

RESULTS AND DISCUSSION

Average weights and observations on feather pigmentation for 4 breeds of chicks fed the basal diet with and without supplemental lysine are given in table 1.

All breeds show a marked growth response to supplemental lysine with the lowest percentage gain in weight, 65%, being recorded for the Black Australorp chicks. This finding suggests that this breed may have a lower requirement for lysine than the others. The statistical significance of this difference in breed response was not tested since the data did not lend

TABLE 1

Growth and feather pigmentation of 4 breeds of chicks fed a lysine-deficient basal diet with and without supplemental lysine

BREED	SUPPLEMENT TO BASAL	NUMBER OF BIRDS AND SEX	AV. WT. AT 4TH WEEK	RESPONSE TO LYSINE	BIRDS SHOWING ACHROMATOSIS OF FEATHERS
			gm	*%*	*%*
	None	20 ♂	208		90
Barred Plymouth		20 ♀	203	97	85
Rock	0.5% L-lysine	20 ♂	424		0
		20 ♀	384		0
	None	19 ♂	172		[1]
Black		20 ♀	181	65	[1]
Australorp	0.5% L-lysine	19 ♂	307		0
		20 ♀	275		0
	None	17 ♂	119		95
Black		11 ♀	106	123	67
Minorca	0.5% L-lysine	20 ♂	266		0
		25 ♀	235		0
	None	22 ♂	132		36
New Hampshire ×		19 ♀	133	126	36
Barred Plymouth	0.5% L-lysine	19 ♂	318		0
Rock		21 ♀	282		0

[1] Count of affected birds was not made. While the achromatosis due to lysine-deficiency was unmistakable the difference from normal was not clear cut since chicks of this breed at 4 weeks of age normally carry some white wing feathers.

themselves to an adequate test. However, support for this hypothesis is provided by a second trial with Black Australorp chicks in which a response of 66% was recorded. All subsequent experiments, for which Barred Plymouth Rock cockerels have been used exclusively, have yielded a response of at least 100%.

For all breeds, the groups receiving the basal diet showed evidence of an inhibition of pigment deposition in the feathers, the groups of male birds being affected more severely than those of females. The achromatosis was particularly evident in the wing feathers, showing usually as a broad band or bar of white. However, in the groups of Barred Plymouth Rock and New Hampshire × Barred Plymouth Rock chicks there were many birds which showed poorly pigmented feathers over most of the body. The symptom appeared in the third week and became most clearly evident when the chicks were 4 weeks of age. Later experiments in which birds were fed the basal diet beyond 4 weeks suggest that maximum severity is reached at 4 weeks. Moreover, the symptom is not associated solely with the use of sunflower seed oil meal since feeding a lysine-deficient basal diet based on wheat gluten rather than sunflower seed oil meal resulted in a severe achromatosis which again was not apparent when supplemental lysine was fed.

Typical birds from all groups were photographed and a few of these are reproduced in figures 1, 2, 3, 4.

In all subsequent studies, Barred Plymouth Rock cockerels have been used, partly because of their apparent susceptibility to the achromatosis, and partly because chicks of this breed are readily obtained commercially. In the many experiments carried out using Barred Plymouth Rock cockerels, the achromatosis has always unmistakably appeared in groups fed the basal diet and failed to appear, or only mildly in the occasional bird, when supplemental lysine was fed.

Several substances reported to be concerned with pigmentation were added to the lysine-deficient basal diet. The achromatosis was not influenced by adding separately, 40 p.p.m. of

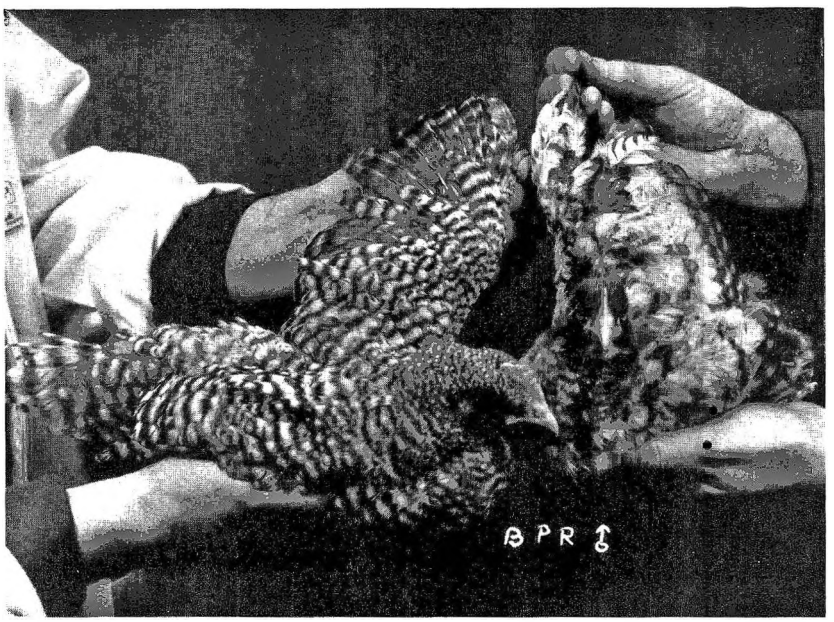

Fig. 1 Four-week-old Barred Plymouth Rock cockerels. Right bird received a lysine-deficient diet.

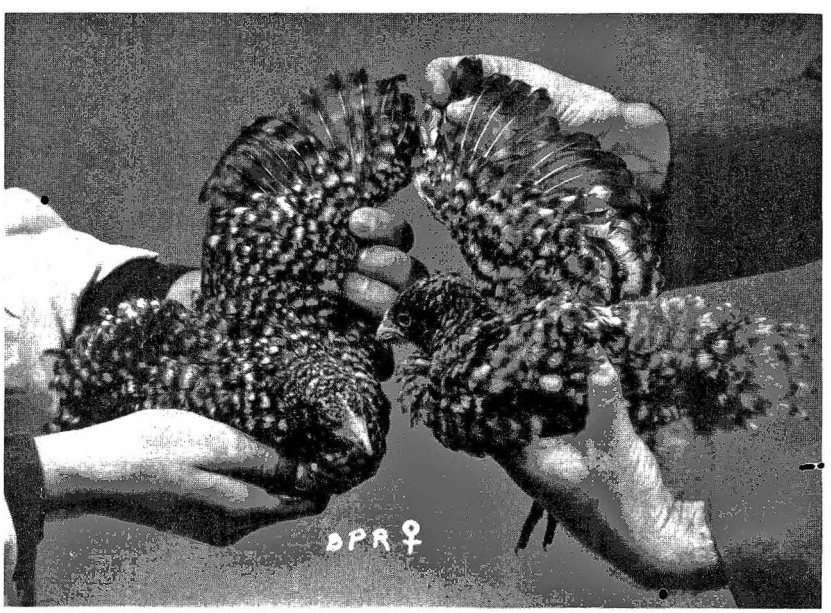

Fig. 2 Four-week-old Barred Plymouth Rock pullets. Right bird received a lysine-deficient diet.

Fig. 3 Four-week-old Barred Plymouth Rock cockerels. Right bird received lysine-deficient diet and shows moderate achromatosis of the feathers.

Fig. 4 Four-week-old Black Minorca cockerels. Left bird received a lysine-deficient diet.

copper (as copper sulfate), 1.25 mg of folic acid or 5.5 mg of iodinated casein per pound of diet. However, the addition of L-tyrosine at a level of 0.5%, while it did not prevent the achromatosis, appeared to reduce somewhat the severity of the symptom.

TABLE 2

Amino acid [1] *and copper* [2] *content of feathers from Barred Plymouth Rock cockerels*

	FROM BIRDS FED BASAL	FROM BIRDS FED BASAL PLUS 0.5% L-LYSINE
	%	%
Crude protein (Nx 6.25)	88.3	88.1
Arginine	5.94	6.08
Histidine	0.62	0.63
Glutamic acid	9.20	9.60
Glycine	7.57	8.10
Isoleucine	4.39	4.56
Leucine	7.31	7.21
Lysine	1.64	1.58
Methionine	0.54	0.52
Phenylalanine	4.41	4.45
Threonine	4.54	4.63
Tryptophan	0.60	0.62
Tyrosine	2.07	2.06
Valine	7.25	7.30
Copper [3]	10.9 p.p.m.	11.3 p.p.m.

[1] Microbiological assay.

[2] Colorimetric biquinoline method.

[3] Copper content of light and dark parts of same feathers 11.5 and 12.0 p.p.m. respectively.

It was considered possible that the consumption of a lysine-deficient diet might alter the structure of the feather protein with a parallel alteration in amino acid composition of the protein to the extent that the deposition of pigment might be affected. To test this hypothesis, feathers from birds receiving the lysine-deficient and the lysine-supplemented basal diet were assayed for amino acid content. Feathers were plucked from 20 4-week-old birds from each group, thoroughly mixed, and a portion withdrawn to provide samples for analysis. These portions were finely cut with scissors. The data of table 2 show only minor differences in amino acid composition be-

tween the normally and abnormally pigmented feathers. Copper analyses were included since copper is an essential component of the enzyme tyrosinase, and since there are several reports that black hair contains more copper than light coloured hair (Sarata, '35; Yoshikawa, '37; Kikkawa, Ogita and Fujito, '55). No difference in copper content of the

TABLE 3

Tests for tyrosinase activity in the primary wing feathers of Barred Plymouth Rock cockerels

DIET	AGE	ACHROMATOSIS	DEGREE OF MELANIN FORMATION	
	days		*optical density* [1]	
Basal + 0.5% L-lysine	35	None	0.280	Average 0.415
	36	None	0.432	
	52	None	0.426 0.476 0.404 0.482	
	56	None	0.398 0.393	
	58	None	0.421 0.404 0.482 0.432	
Basal	35	Severe	0.076 0.025	Average 0.130
	58	Severe	0.056 0.215	
	58	Mild	0.268	
	63	Severe	0.076 0.168	
	63	Mild	0.328	
	72	Severe	0.032	
	72	Moderate	0.066	
	72	Mild	0.125	

[1] Each value represents the average of at least two determinations on a single bird.

feathers was found between the composite samples or between light and dark portions of the same feathers taken from birds fed the lysine-deficient diet.

It has not been shown that severe deficiencies of essential amino acids other than lysine will not produce feather achromatosis. Consequently, it cannot be claimed that the symptom is unique for lysine deficiency as far as amino acids are concerned. Preliminary experiments have shown that the level of food intake, rate of growth, or the level of protein intake, provided the protein is balanced with respect to lysine, are not the major factors concerned.

Results of the tests for tyrosinase activity in the feathers are given in table 3. They are given as optical densities which should be directly proportional to the concentration of pigment in solution provided that the Lambert-Beer law is being followed over the range of concentration involved.

Among the birds fed the basal diet there is a range of activity from almost zero to fairly active as judged by the values obtained with the normally pigmented birds. To some degree the measured tyrosinase activity follows the severity of the achromatosis.

It will be noted that birds tested were of various ages. Furthermore, the birds fed the lysine-deficient basal diet were considerably lighter in weight, and possessed smaller feathers than their counterparts fed the lysine-supplemented diet, even though the former were, on the average, older when sampled.

These factors might be a source of some variation in measurements between the two groups. Nevertheless, it is believed by the authors that these factors could not explain the wide differences observed, and that this difference, while not strictly quantitative, shows that tyrosinase activity, as measured by the method outlined, is considerably less in achromatous than in normally pigmented feathers. Whether this difference in activity can be attributed to a smaller concentration of enzyme or to the presence of an inhibitor or deficiency of an activating substance in the feathers of lysine-deficient birds has not been determined.

SUMMARY

Four breeds of chicks, Barred Plymouth Rock, Black Minorca, Black Australorp and a New Hampshire × Barred Plymouth Rock cross developed an achromatosis of the feathers when fed a lysine-deficient diet. Cockerels were more severely affected than pullets. The symptom was not prevented by supplementing the diet with folic acid, iodinated casein, copper or tyrosine. The amino acid composition of achromatous feathers and normally pigmented feathers was

similar. However, the tyrosinase activity of feather quill homogenates, as measured by pigment production on incubation with tyrosine, was less in achromatous than in normal feathers.

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THE INFLUENCE OF LIPOTROPIC FACTORS ON THE PREVENTION OF NUTRITIONAL EDEMA IN THE RAT [1]

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It has been previously reported that severe anemia and nutritional edema could be produced in both weanling and adult rats by feeding a diet deficient in choline and low in protein (Engel, '48; Alexander and Engel, '52). The edematous condition was associated with fatty infiltration and cirrhosis of the liver.

Schaefer, Salmon and Strength ('49) established the existence of an interrelationship between vitamin B_{12} and choline or methionine. The addition of vitamin B_{12} to diets low in choline and methionine reduced the incidence and severity of renal damage and produced an increased gain in weight of weanling rats. Later Schaefer et al. ('50) showed that folacin, vitamin B_{12}, choline and methionine were interrelated. Preliminary observations made by Engel and Alexander ('52) indicated that vitamin B_{12} and folacin would prevent anemia and nutritional edema development in rats fed low-protein, choline-deficient diets. A supplement of folacin alone appeared to be without effect in the prevention of the anemic and edematous condition.

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This paper is concerned (1) with an extension of findings on edema-prevention with choline, vitamin B_{12} and folacin and (2) with an investigation to determine the effect of other lipotropic agents and related compounds on the edema process.

EXPERIMENTAL

Weanling rats of the Sprague-Dawley strain were used in this study. They were uniformly grouped with respect to sex, weight and litter and were housed individually on wire

TABLE 1

Percentage composition of the basal diets[1]

INGREDIENTS	DIET			
	C-189	C-223	C-303	C-336
Methanol-extracted peanut meal	10.0	7.0	..	..
Methanol-extracted casein	2.0	1.5	..	..
Oxidized casein	..	..	7.0	..
Salts[2]	4.0	4.0	4.0	4.0
Sucrose	63.9	67.4	68.8	91.0
Lard	19.0	19.0	19.0	4.0
Cod liver oil	1.0	1.0	1.0	1.0
L-Cystine	0.1	0.1	0.1	..
DL-Tryptophan	..	..	0.1	..

[1] The following vitamins were added, in milligrams per kilogram of diet: Thiamine, 6; riboflavin, 6; pyridoxine, 6; inositol, 1,000; calcium pantothenate, 30; niacin, 25; 2-methyl-1, 4-naphthoquinone, 5; biotin, 0.5; alpha-tocopherol, 50 and alpha-tocopherol acetate, 50. Control animals received a further supplement of 2,000 mg of choline chloride per kilogram of diet.

[2] Salmon, J. Nutrition, *33:* 155, 1947.

screens in an air-conditioned animal room with food and water given ad libitum. The percentage composition of the basal diets used is given in table 1. Rats that received the edema-inducing diets were given an initial 0.1% choline chloride supplement that was withdrawn after two weeks. Control animals received the same diet supplemented with 0.2% choline chloride for the entire experimental period. Animal weights were recorded weekly.

Hemoglobin determinations were made by the oxyhemoglobin method of Evelyn ('36). Liver fat determinations were made in one series of animals. The dried, pulverized liver sample was extracted with anhydrous ether in a Nolan extractor.[2] Fat was expressed as total ether-extractable material.

RESULTS

For evaluation of the results in these studies, generalized edema denotes that ascites and hydrothorax were accompanied by a well-defined accumulation of fluid in the subcutaneous tissues. Fatty and cirrhotic livers were always found in the animals with edema.

Preliminary results given in table 2 (experiment 1) indicate that a combination of vitamin B_{12} and folacin as a supplement to a 7% protein (peanut meal-casein), choline-deficient diet will prevent the development of anemia and nutritional edema in rats, even though the livers from two rats were cirrhotic. The supplement of vitamin B_{12} alone afforded considerable protection but was nevertheless inferior to the combined supplement of this vitamin and folacin. Vitamin B_{12} alone permitted one case of edema in 7 rats over an experimental period of 119 weeks; six of these 7 rats had cirrhotic livers at death. Four of the 7 rats showed a gradual decline of hemoglobin during the time they survived. These 4 animals died before the 63rd week of the experiment, thus accounting for the increased level of hemoglobin at 63 weeks for the three survivors. Survival time was also considerably lengthened in animals receiving vitamin B_{12} and folacin in combination, as compared with rats receiving the basal diet or the same diet supplemented with vitamin B_{12} alone or folacin alone. Folacin fed singly did not give protection against nutritional anemia or edema. Six of 7 rats receiving the basal low-protein, choline-deficient diet developed generalized edema in an average of 12 weeks and had a hemoglobin level of 4.2 gm/100 ml of blood just prior to death.

[2] Nolan ('49).

Data from an extension of the aforementioned study, including choline and methionine supplements as well as vitamin B_{12} and folacin, are presented in table 2 (experiment II). Even though an initial 0.1% choline chloride supplement was added to the diet for a two-week period, approximately 50% of the animals receiving the unsupplemented diet died as a result of kidney hemorrhage in 7 to 14 days after the choline supplement was removed. Animals that died of kidney hemorrhage were not included in the compilation of the data. Choline alone, methionine alone or a combination of vitamin B_{12} and

TABLE 2

Influence of choline, methionine, vitamin B_{12} and folacin on the prevention of edema in weanling rats fed a 7% protein diet

COMPOUND ADDED TO CHOLINE-DEFICIENT BASAL DIET	AVERAGE BODY WEIGHT AT			HEMOGLOBIN VALUES AT		NO. WITH KIDNEY HEMORRHAGE	NO. WITH GENERALIZED EDEMA	AV. SURVIVAL TIME [1]
	0 wks.	50 wks.	63 wks.	50 wks.	63 wks.			
	gm	*gm*	*gm*	*gm%*	*gm%*			*wks.*
Experiment I								
None (Diet C-189)	62	...	...	4.2 [2]	...	1	6/7	12
Folacin [3]	67	...	...	3.9 [2]	...	1	5/7	15
Vitamin B_{12} [3]	60	255(3) [4]	291(3)	10.4	13.2	0	1/7	47
Vitamin B_{12}, folacin	59	214(6)	218(4)	13.0	13.3	0	0/7	70
Experiment II								
None (Diet C-189)	49	...	...	4.4 [2]	...	4	3/7	11
Choline [3]	48	158(3)	186(2)	14.0	15.2	0	0/8	50
Folacin	49	...	...	3.5 [2]	...	4	4/8	11
Vitamin B_{12}	50	218(4)	211(3)	11.8	14.3	3	0/8	53
Vlitamin B_{12}, folacin	50	238(4)	260(2)	13.9	15.4	1	0/8	52
Choline, vitamin B_{12}, folacin	48	258(3)	264(3)	14.5	14.7	0	0/8	48
DL-Methionine [3]	52	237(5)	247(3)	14.3	14.8	0	0/8	54

[1] This value does not include animals that died due to kidney hemorrhage.

[2] Average values obtained one to two days prior to death for rats that developed edema.

[3] Folacin was added at 2 mg/kg of diet; vitamin B_{12} was added at 30 μg/kg of diet; methionine was added at 6 gm/kg of diet; and choline chloride was added at 2 gm/kg of diet.

[4] Figures in parentheses are the number of animals on experiment at time indicated.

folacin, or of vitamin B_{12}, folacin and choline were equally effective in the maintenance of normal hemoglobin levels and in the prevention of edema. Vitamin B_{12} alone also prevented the development of generalized edema and liver cirrhosis. However, hemoglobin declined below normal levels in approximately one-half of the vitamin B_{12}-supplemented animals. Choline alone was inferior to the other supplements with respect to growth promotion. All of the rats that received

TABLE 3

Influence of choline, methionine, vitamin B_{12} and folacin on the prevention of edema in weanling rats fed a 5% protein diet

COMPOUND ADDED TO CHOLINE-DEFICIENT DIET (Diet C-223)	AVERAGE BODY WEIGHT AT			HEMOGLOBIN VALUES AT		NO. WITH GENERALIZED EDEMA	AV. SURVIVAL TIME
	0 wks.	30 wks.	70 wks.	30 wks.	70 wks.		
	gm	*gm*	*gm*	*gm%*	*gm%*		*wks.*
None	43	64 [1](10)[2]	...	8.3 [1]	...	10/12	16
Choline [3]	43	80(8)	...	11.6	...	0/8	34
Vitamin B_{12} [3]	44	90(5)	163(3)	9.4	13.3	4/8	57
Vitamin B_{12}, folacin [3]	43	190(7)	202(4)	12.7	13.0	0/7	71
Vitamin B_{12}, folacin, choline	45	92(8)	188(4)	12.5	13.6	0/8	49
DL-Methionine [3]	44	101(6)	188(6)	11.7	13.7	0/7	82

[1] This value was for 12 weeks, approximately 14 days prior to development of edema in the majority of the animals.

[2] Figures in parentheses are the number of animals on experiment at this time.

[3] Choline chloride was added at 2 gm/kg of diet; vitamin B_{12} was added at 30 μg/kg of diet; folacin was added at 2 mg/kg of diet; and methionine was added at 6 gm/kg of diet.

the basal low-protein, choline-deficient diet or the same diet supplemented with folacin developed anemia and edema, and died within 11 weeks.

Results given in table 3 show that even when the protein level was lowered to 5% in the choline-deficient diet (basal diet no. C-223), supplements of choline alone, methionine alone, or a combination of vitamin B_{12} and folacin, or of vitamin B_{12}, folacin and choline were effective in the prevention of nutritional edema and in the maintenance of near-

normal hemoglobin levels in the rats. Vitamin B_{12} alone was not as effective as the aforementioned supplements in that 4 of 8 rats developed generalized edema and hemoglobin levels were usually lowered. Ten of 12 animals that received the basal 5% protein, low-choline diet developed generalized edema in an average of 16 weeks and were anemic at the time of death. Under conditions of this experiment, methionine alone or a combination of vitamin B_{12} and folacin, or of vitamin B_{12}, folacin and choline promoted more growth than did choline or vitamin B_{12} fed singly.

When wheat gluten was used in another study as a source of protein in choline-deficient diets fed to weanling rats, it was necessary to lower the fat and increase the protein content from the level that had been used in the previous work. Even when the diet contained 8% gluten and 10% fat along with supplements of choline, or methionine, or vitamin B_{12} and folacin, or a combination of these, the animals gained only about 1 gm/week for a 66-week experimental period. Choline alone, methionine alone, or a combination of vitamin B_{12} and folacin, or of choline, methionine, vitamin B_{12} and folacin were equally effective in the prevention of edema development and in the maintenance of near-normal hemoglobin levels. Generalized edema accompanied by a lowered hemoglobin concentration developed in three of 7 rats receiving the basal choline-deficient diet in an average of 31 weeks. Liver fat (dry basis) was considerably higher than normal in all groups (39.6 to 56.3%, dry weight basis).

Data are presented in table 4 from a study to determine the effect of glycine, methionine, choline, vitamin B_{12} and folacin when added singly or in combination to a protein-free diet and fed to male adult rats. Results show that all groups of animals that received this diet lost approximately 50% of their original body weight during the first 12 weeks of the experiment regardless of the vitamin or amino acid supplement used. Hemoglobin levels declined at the same rate in all groups of rats through the first 10 weeks of the experi-

TABLE 4

The effect of lipotropic supplements when fed to adult rats on a protein-free diet

ADDITIONS TO PROTEIN-FREE BASAL DIET (Diet C-336) [1]	AV. BODY WEIGHT		AV. HEMOGLOBIN				NO. WITH GENERALIZED EDEMA	AVERAGE SURVIVAL TIME
	Initial	12 wks.	10 wks.	12 wks.	18 wks.	20 wks.		
	gm	*gm*	*gm%*	*gm%*	*gm%*	*gm%*		*wks.*
None	330	163	11.6	8.8	..	..	1/6	14
Choline	321	169	12.4	9.8	..	..	0/6	12
Folacin	321	170	12.4	11.3	8.9	7.3	0/6	18
Vitamin B_{12}	333	166	11.9	11.0	7.8	6.3	0/6	16
Vitamin B_{12}, folacin	328	180	11.7	11.3	10.2	8.1	0/6	21
Choline, vitamin B_{12}, folacin	319	174	12.6	11.9	9.7	8.8	0/6	22
DL-Methionine, 0.4%	323	190	10.9	10.2	7.0	6.7	0/6	18
Glycine, 3%	332	170	10.2	10.2	..	..	0/6	11
Glycine, 3%, choline, vitamin B_{12}, folacin	334	183	12.1	12.4	9.0	8.4	0/6	21
Fat increased to 20%	327	172	11.6	9.6	5.6	..	0/6	16
Fat increased to 20%, choline	312	169	12.0	10.8	..	..	0/6	15
Casein, 20%	328	417	16.0	16.1	15.9	15.8	0/6	> 26
Casein, 20%, choline, vitamin B_{12}, folacin	307	426	15.9	15.9	16.2	16.2	0/3	> 26

[1] Choline was added at 2 gm/kg of diet; vitamin B_{12} was added at 50 μg/kg of diet; folacin was added at 2 mg/kg of diet.

mental period. However, thereafter vitamin B_{12} and folacin or vitamin B_{12}, folacin and choline in combination were the most effective supplements for hemoglobin maintenance in the rats on the protein-free diet. A combination of vitamin B_{12} and folacin was more effective in the prolongation of life in the protein-deficient animals than was the single addition to the diet of choline, glycine, methionine, folacin or vitamin B_{12}. An increase to 20% in the fat level of the protein-deficient diet had no effect on hemoglobin levels or survival time. However, generalized edema developed in only one animal which received the basal protein-free, choline-deficient diet (C-336). Six rats that received a 20% casein supplement made an average weight gain of 89 gm for the first 12 weeks of the experimental period, while three rats that received this level of casein plus choline, vitamin B_{12} and folacin made an average gain of 119 gm.

Data have been obtained from a study to ascertain the effect on edema prevention of betaine and dimethylaminoethanol as a supplement to a 7% protein (peanut meal-casein), choline-deficient diet and the effect of homocystine, vitamin B_{12} and folacin as a supplement to a 7% oxidized casein, choline-deficient diet fed to weanling rats (table 5). The supplements of betaine or dimethylaminoethanol alone, or homocystine, vitamin B_{12} and folacin in combination permitted growth and were effective in the maintenance of normal hemoglobin levels in the rats. A combination of homocystine, vitamin B_{12} and folacin, or dimethylaminoethanol alone, or betaine alone prevented development of generalized edema. Even though betaine alone and the combination of homocystine, vitamin B_{12} and folacin afforded complete protection against edema development, hepatic cirrhosis was noted in some animals (4 in the betaine group and one in the homocystine, vitamin B_{12} and folacin group). When homocystine alone was added to the oxidized-casein, choline-deficient diet, 4 of 5 rats developed generalized edema; two of 5 animals receiving the basal low-protein, choline-deficient diet (peanut meal-casein) developed generalized edema.

In another study, the effect of dietary fat level on edema production in weanling male rats receiving a 7% protein, choline-deficient diet was investigated. Data from this 60-week study showed that when the low-protein, choline-deficient diet contained 7 to 30% of dietary fat, the incidence of edema increased above that produced by a comparable diet that contained less than 7% of dietary fat. Increased dietary fat caused a decreased food intake and thus a corresponding

TABLE 5

The influence of betaine, dimethylaminoethanol and homocystine on edema prevention in weanling rats

DIET	AVERAGE BODY WEIGHT AT		TERMINAL HEMOGLOBIN	NO. WITH GENERALIZED EDEMA	AVERAGE SURVIVAL TIME
	0 wks.	50 wks.			
	gm	*gm*	*gm%*		*wks.*
7% Protein (C-189)[1]	51	...	4.7	2/5	17
C-189 + 0.3% Betaine·HCl	56	217	13.4	0/5	> 79
C-189 + 0.3% DME[2]	55	180	12.6	0/6	> 79
7% Oxidized casein (C-303) + 0.4% DL-homocystine	58	...	5.6	4/5	17
C-303 + 0.4% DL-homocystine + vitamin B_{12}[3] + folacin[3]	51	241	12.8	0/4	> 79

[1] The protein in this diet is furnished by peanut meal and casein.

[2] Dimethylaminoethanol.

[3] Vitamin B_{12} was added at 30 μg/kg of diet; folacin was added at 2 mg/kg of diet.

decrease in protein intake. The time required to produce edema decreased as the fat level of the diet was increased. Hemoglobin levels in the choline-deficient rats were near normal at the end of the first 15 weeks if the fat level was 2 to 10% of the diet. At this same time, comparable animals receiving 15 to 30% of dietary fat were very anemic. However, by the end of the 35th week, the choline-deficient animals receiving diets with low levels of fat had also become anemic. Choline was effective for the maintenance of normal hemo-

globin levels and afforded complete protection against edema development regardless of the fat level used.

The data presented in table 6 show that choline will prevent edema development in weanling rats even when the methionine content of the diet is as low as 0.02%. Some increases in weight gains and hemoglobin concentrations were noted in

TABLE 6

Effect of vitamin B_{12} and folacin in a 7% oxidized casein diet fed to weanling rats [1]

COMPOUND ADDED TO CHOLINE-DEFICIENT BASAL DIET	NO. OF RATS	METHIONINE ADDITION TO DIET	AVERAGE BODY WEIGHT AT			AVERAGE HEMOGLOBIN		AV. SURVIVAL TIME
			0 wks.	8 wks.	36 wks.	15 wks.	36 wks.	
		%	*gm*	*gm*	*gm*	*gm%*	*gm%*	*wks.*
Choline [2]	6	0.02	55	39(5) [3]	...	...	...	9
Choline, vitamin B_{12}, folacin [2]	6	0.02	54	42(5)	...	12.3(3)	...	15
Choline	6	0.04	53	43(4)	...	12.0(2)	...	10
Choline, vitamin B_{12}, folacin	6	0.04	49	46(5)	...	12.5(2)	...	15
Choline	6	0.08	55	58(6)	95(2)	13.8(6)	15.7	> 36
Choline, vitamin B_{12}, folacin	6	0.08	53	62(6)	127(6)	14.7(6)	15.4	> 36

[1] Basal diet C-303.

[2] Choline chloride was added at 2 gm/kg of diet; vitamin B_{12} was added at 30 μg/kg of diet; and folacin was added at 2 mg/kg of diet.

[3] Figures in parentheses are the number of animals on experiment at this time.

the animals as an apparent result of the methionine-sparing effect of vitamin B_{12} and folacin. Vitamin B_{12} and folacin also increased the longevity of the rats receiving the lowest levels of methionine compared with those receiving choline as the supplement.

DISCUSSION

The importance of choline and some possible modes of action of this compound as an edema-preventive have been

discussed in a previous paper (Alexander and Engel, '52). More recently, lipotropic substances and the antidiuretic hormone, pitressin, have been shown to be closely associated with each other in the normal and pathological physiology of fluid metabolism in the rat. Lloyd and Loewy, reported by Lloyd ('52), have shown that when rats have definite liver damage, the administration of small doses of pitressin results in a marked water retention that is not evidenced by normal control animals which have no liver damage. These damaged livers were produced by feeding a diet deficient in both protein and lipotropic substances. Animals that received the same level of protein supplemented with choline, vitamin B_{12} and folacin served as normal controls. Guggenheim and Diamant ('55) also found that choline-deficient rats possess a diminished pitressin-inactivating ability. These workers showed that vitamin B_{12}, as well as choline, restored the impaired ability of the liver to destroy pitressin. Rats from the present study invariably had fatty infiltration and cirrhosis of the liver prior to the development of edema. This relationship of damaged liver and decreased ability to inactivate pitressin emphasizes the importance of lipotropic substances as edema-preventives.

The important role that vitamin B_{12} and folacin assume in the biological synthesis of choline and methionine must be considered as a factor in the prevention of nutritional edema. It has been known for some time that choline and methionine methyl groups as well as the ethanolamine moiety of choline can be formed from nonessential dietary constituents (du Vigneaud, '50; Stekol, '50). It was concluded by Schaefer and Knowles ('51) that the sparing action of vitamin B_{12} and folacin can be attributed to the effect of these vitamins in stimulating the biological synthesis of choline and methionine. In the present experiment, when comparison is made between hemoglobin levels maintained by rats receiving a low-protein, choline-deficient diet supplemented with vitamin B_{12} and folacin and by rats receiving the same diet supplemented with

either choline or methionine, it is seen that the values are almost the same. In addition, results of this experiment show that a combination of vitamin B_{12} and folacin added to the same low-protein, choline-deficient diet has been as effective in the prevention of edema, although not as effective in prevention of cirrhosis, as has been choline or methionine. Therefore, the preceding explanations of the influence of vitamin B_{12} and folacin are very possible and feasible ones.

The pathology of edematous rats in the present experiment resembles in many respects that in human cases of kwashiorkor and sindrome policarencial infantil (nutritional syndromes commonly found among indigenous African and Central American children). Brock and Autret ('52) charterize the syndrome of kwashiorkor as consisting of the following clinical signs: (a) retarded growth and gross atrophy of the muscles, (b) edema, usually associated with hypoalbuminemia, (c) fatty infiltration, cellular necrosis, or fibrosis of the liver, and (d) normocytic or macrocytic anemia. Autret and Behar ('54) describe sindrome policarencial infantil as being essentially the same syndrome as kwashiorkor, but point out that the severe form seems to be less frequent in Central America than in Africa, probably because cases are diagnosed earlier in Central America. One of the most important features of the clinical picture in children of this locality is the edema that always accompanies the syndrome and is usually the abnormal sign that attracts the attention of parents to it. Even though a deficiency of dietary protein, particularly animal protein, is considered to be the primary cause of this syndrome, it should be pointed out that avitaminosis may also be involved. Therefore, it is possible that a deficiency of lipotropic substances might be assuming a major role as an etiological factor of kwashiorkor.

SUMMARY

The edema observed in animals fed a protein deficient diet was prevented by the methyl compounds, choline, dimethyl-

aminoethanol, betaine and methionine. Vitamin B_{12} was also effective, and its action was increased by the addition of folacin, which alone was without effect. Longevity and body weight of rats were also improved by a supplement of vitamin B_{12} and folacin and to a lesser extent by choline.

The resemblance of the pathology of the edematous rats to that in human cases of kwashiorkor has been discussed.

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EFFECTS OF STORAGE UPON THE NUTRITIVE VALUE OF BARLEY GRAIN AS A SOURCE OF PROTEIN

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Data concerned with the chemical and nutritional changes in stored cereal grains have been summarized in a number of reviews of the literature (Jones et al., '42; Nutrition Reviews, '43; Watson, '46; Zeleny, '48). However the evidence available is often inconclusive and in some instances even contradictory.

Takahashi and Kiyosha ('28), using chemical methods, were able to show that major chemical changes occurred in barley protein during storage. Jones and Gersdorff ('41), after a detailed chemical study, reported major changes in solubility and in *in vitro* digestibility of the proteins of wheat during storage. In a later study these same authors (Jones et al., '42) found that storage led to similar changes in corn protein. The corn study was expanded to include a rat feeding trial and it was concluded that utilization of the corn protein measured thus was also reduced by storage.

Mitchell and Beadles ('49), after a very detailed study with rats, concluded that the proteins of corn and wheat showed no changes in digestibility or biological value after three years of storage. Cabell and Ellis ('55) concluded that storage reduced the utilization of corn protein by rats in one case but not in another.

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Feeding trials with large animals have generally shown that storage, under adequate dry conditions, does not change the value of the cereal grains, either as a source of energy or as a source of protein, in well-balanced rations (Culbertson et al., '42; Longwell, '45; Aubel, '55).

The experiments reported herein were designed to study, by biological methods, the effects of prolonged storage upon the proteins of barley grain. Barley grain from 4 successive crop years both before and after storage was fed to growing rats. Data for food consumption, weight gain, and nitrogen balance were used as the criteria of measurement in the rat study. In a related study, barley which had been in storage for three years was compared to fresh barley as the chief constituent of the ration of growing swine. Criteria of measurement were the weight gains and the food consumption for individuals receiving the stored or fresh barley at two different levels of protein supplementation and from two different protein sources.

EXPERIMENTAL

Rat experiment. For 4 consecutive years samples of barley (Atlas 46 variety) grown on the University farm were collected for this study. Part of each sample was fed immediately as fresh barley. The remainder was stored for use in future years. The barley was stored as whole grain in 30-gallon galvanized metal garbage cans. These were covered but not sealed, and stored in a room where the temperature and humidity fluctuated with the outside environment. Environmental temperature ranged from a low of 30°F. to a high of 110°F. while relative humidity ranged from about 10% to nearly 100%. A piece of absorbent cotton soaked in carbon tetrachloride was placed in each can every three months to prevent insect infestation. Table 1 shows the chemical analysis of each of the barley samples collected in different years. Analyses made of the same sample in different years gave results which were always essentially the same.

Four separate experiments were conducted, one each year, soon after the barley was harvested, during the summers of

1952, 1953, 1954, and 1955. Barley used in the 1952, 1953 and 1954 studies was tested at three different dietary protein levels: (1) barley as the sole source of protein, (2) barley at a level to furnish 60% of the protein plus an additional 4% of casein to give a total content of 10% of protein and (3) barley at a level to furnish 37.5% of the protein plus an additional 10% of casein to give a total content of 16%. This was expected to give a measure of barley as a sole source of protein and of the supplemental value of barley protein when fed with casein. In the 1955 study the different barleys were compared only at the (2) level, namely, barley plus casein to give a 10% dietary protein level. At each dietary level the amount

TABLE 1

Analysis of different barleys (D.M. basis)

CATEGORY OF INTEREST	1952	1953	1954	1955
Dry matter	91.50	91.46	91.70	91.80
Protein (N × 6.25)	8.42	8.99	9.95	12.10
Ether extract	1.77	2.13	2.30	2.18
Crude fiber	6.80	6.86	7.12	5.50
Ash	3.20	3.22	3.05	2.67

of protein from barley was kept constant, being equal to that in the barley of lowest protein content. Thus the total amount of barley in the rations of the several crop years differed because of variation in protein content. Sucrose was the variable component used to replace barley to keep the protein level constant. Within rations of a given protein level the amount of casein was constant. Each year one group of rats at each protein level was fed a purified diet with casein as the only source of protein. These groups were considered as control groups for comparisons between years. The casein used was from a fresh supply each year and therefore was not the same in all feeding trials.

The rations used were based on that used by Meyer ('54). The constant components of the rations were cottonseed oil,

5%, salts, 4% and ample amounts of added vitamins. The variable components were barley, casein, and sucrose. Alpha tocopherol was added to all rations as an anti-oxidant. The barley was ground through a 1/32 inch screen in a hammer mill immediately before it was mixed into a ration.

Inbred Long-Evans rats from the colony of the Animal Husbandry Department were used in all the trials. Weanling male rats were housed individually in wire-bottom cages and fed the test rations for periods of 4 weeks. In the 1952 and 1953 trials 6 rats were included in each test group; eight rats were used in each test in the 1954 and 1955 trials. Special scatter-proof food cups were used. Weekly weight gain and food consumption figures were recorded. The animals received food and water ad libitum.

During the second and third week of each trial the rats were confined in individual metabolism cages for complete collection of urine and feces. The metabolism cage used was designed from the description of Harned et al. ('49). During the course of the study animals which showed definite evidence of respiratory infection were eliminated from the trial.

SWINE EXPERIMENT

This study was factorially designed to determine if there was a difference in the nutritive value of the protein of barley which had been stored for three years and that which had been freshly harvested. The barley used was similar to that employed in the rat trials. The 1952 barley had been stored in a steel tank for three years; the 1955 material was freshly harvested and sacked. Both barleys were ground through a 3/8 inch screen in a hammer mill just before the feeding period began.

The two barleys differed in protein content (N $\times$ 6.25). The 1952 barley contained 9.6% total protein; the 1955, 11.4%. The actual composition of the diets is shown in table 2.

Twenty-four weanling Hampshire barrows and gilts averaging 43 pounds in weight were used in the study. They had

been treated for worm infestation with cadmium oxide and vaccinated against hog cholera prior to the start of the trial. The pigs were allotted into 8 groups of three animals each. Four groups received each of the barleys — two groups at the 10% level of total protein and two at the 16% level. At each protein level within barleys one group received casein as the supplemental protein and the other received a 60/40 mixture

TABLE 2

Basal diets used in the swine study

COMPONENT	1952 BARLEY DIET	1955 BARLEY DIET
	%	%
Barley	78.00	66.2
Sucrose	Variable	Variable
Casein	3.12 or 10.34	3.12 or 10.34
Soybean meal	3.74 or 12.00	3.74 or 12.00
Cottonseed meal	2.50 or 8.00	2.50 or 8.00
Mineral mix[1]	2.00	2 00
Vitamin mix[2]	0.2	0 2

[1] The composition of the mineral mixture was as follows: 40.0 parts ground oyster shell, 31.2 steamed bone meal, 25.0 iodized salt, 2.0 $MgCO_3$, 1.0 $FeSO_4H_2O$, 1.10 $CoCl_3 \cdot 6H_2O$, 0.1 $CuSO_4$, 0.04 $ZnCO_3$.

[2] The vitamin mix supplied 100 mg of riboflavin, 500 mg of pantothenic acid, 600 mg of niacin, 500 μg of vitamin B_{12}, 85,000 units of vitamin A and 10,900 units of vitamin D to each 100 pounds of feed.

of soybean oil meal and cottonseed oil meal (solvent extracted).

The pigs were individually hand-fed twice daily. Water was available from automatic waterers during the time that the animals were eating (two- 2-hour periods per day). The animals were fed as much as they would eat without wasting feed. Throughout the trial they were kept on a concrete floor within the experimental barn. Individual feed consumptions and body weight gains were determined for each two-week period during the study.

RESULTS AND DISCUSSION

Rat experiment

A study of the results summarized in table 3 indicate that storage had no measureable effect upon the nutritive value of the barley protein under the conditions prescribed. The values for percentage of retained nitrogen and for average gain were analyzed within years using the method of analysis of variance (Snedecor, '46). In no case was a significant difference between barleys indicated.

Although the rats used in different years were from the same highly inbred colony, many environmental factors were not completely controlled between years. Thus any comparisons between years should be made with caution. However, a purified diet in which crude casein was the only source of protein was fed each year to allow a standard for comparison of response between years. It must be remembered, however, that fresh crude casein was used each year and naturally would be from a different source. Nitrogen retention values showed remarkable agreement between different years. However, the weight gain on the casein diets differed somewhat from year to year.

The diets fed at the 6% level (barley without added casein) are the only ones in which barley protein alone was fed. A cursory examination of the results of the 1953 trial suggest that the 1952 barley may have deteriorated in storage. However the 1952 and 1953 barleys fed in 1954 were from the same samples used in 1953, in this case no differences were indicated. Fresh and stored barleys gave approximately the same results in the 1954 study when fed as the only source of protein.

It should be noted that in the diets in which all protein came from barley alone, the protein level was very low (approximately 6%). This low dietary level may have affected the results in the case of the barley diets and also in the case of the purified diet. Mitchell ('24) has shown that the biological value of proteins is almost always higher when the determina-

TABLE 3

Results of barley protein studies conducted in 4 consecutive years

YEAR	DIET	NITROGEN RETENTION			4-WEEK WEIGHT GAIN		
		6% protein level [1]	10% protein level	16% protein level	6% protein level [1]	10% protein level	16% protein level
		%	%	%	*gm*	*gm*	*gm*
1952 [2]	1952 barley	..	..	..	12	110	164
	Purified [3]	..	..	..	17	74	145
1953	1952 barley	38	58	62	18	93	145
	1953 barley	47	60	62	23	105	160
	Purified	67	68	63	29	90	160
1954	1952 barley	39	56	54	14	95	157
	1953 barley	37	56	54	10	98	156
	1954 barley	39	58	54	12	110	169
	Purified	59	68	61	26	80	169
1955	1952 barley	..	56	..	..	78	...
	1953 barley	..	57	..	..	90	...
	1954 barley	..	58	..	..	89	...
	1955 barley	..	58	..	..	83	...
	Purified	..	64	..	..	65	...

[1] Barley furnished 100, 60 and 37.5% respectively of the protein at the 6, 10, and 16% protein levels; casein furnished the remainder of the protein at 10 and 16% protein levels.

[2] Six rats were fed each diet in 1952 and 1953 while 8 rats were fed each diet in 1954 and 1955.

[3] Casein was the sole source of protein in the purified diets.

tion is made at the 5%, rather than at the 10% level, the usual level employed. The protein level of the diets used here was determined by the amount of protein present in the barleys. Since at each protein level the amount of barley protein was kept constant, the barley of lowest protein content actually determined the amount of barley protein in each diet.

The diets fed at the 10% level (barley plus casein to give 10% total protein) were fed through 4 years (1952, 1953, 1954 and 1955). The results obtained indicate no change in the barley samples as storage time increased. Results obtained were actually a measure of the utilization of a mixture of proteins rather than of a single protein. Since the amounts of casein and barley protein were kept constant within diet groups, the values can be used to compare the supplemental value of barley from different years. Apparently the barley protein has not lost any of its supplemental protein value even after three years of storage, when fed in the 10% protein diet.

Results from diets fed at the 16% protein level (barley plus casein to give 16% total protein) also indicate no loss of supplemental value after two years of storage.

In all diet groups the net nitrogen balance remained positive. Likewise, all animals on the three different protein levels gained weight during the 4-week trial period. Mitchell ('44) has summarized data indicating that live weight increase is not a reliable measure of protein nutritive value. However, live weight gain is almost always measured, and it is certainly indicative of a positive nutritive condition. The data for weight gain are presented here as supporting evidence and also as an indication of the nutritive level of the over-all study.

The results reported in this study agree in general with those obtained by Mitchell and Beadles ('49) in their biological study of corn and wheat proteins. They do not necessarily conflict with results reported by Takahaski and Kiyosha ('28) or Jones and Gersdorff ('41) or Jones et al. ('42). The chemical changes noted by these workers do not necessarily indicate a change in the nutritive value of the proteins. This

is further indicated by the fact that total nitrogen and free ammonia remained constant in the grain samples as storage time increased (Jones et al., '42).

Jones et al. ('42) and Cabell and Ellis ('55) have reported rat feeding trials in which it was concluded that storage decreased the nutritive value of corn proteins. The reduced growth-promoting power of stored corn, as reported by Jones et al. ('42) may have been due merely to reduced food intake; they have pointed out this fact but conclude that it is irrelevant so far as feeding value is concerned. Live weight gain and food consumption were the only measurements made to measure the effect of storage on corn grains by Jones et al. ('42) in feeding rats in different seasons and different years.

Cabell and Ellis ('55) have also used live weight gain and food consumption as their criteria of measurement. In their study of the nutritive value of protein, the corn diets were supplemented with lysine and tryptophan. Thus they were not necessarily studying corn *per se*. Corn samples were collected from three areas some distance apart. All samples were field dried yellow corn but no information was available about the varieties represented. Commercial grades of the corn samples varied all the way from 1 through 5 to sample grade. It is interesting to note that corn which had been stored for two years gave a greater weight gain than that which had been stored only one year. Also, one sample of corn which had been stored for 6 years gave a greater weight gain than the sample stored for one year.

Swine experiment

Average daily weight gains and feed efficiency values for the swine study are summarized in table 4. The study was designed to determine whether or not storage had any effect upon the nutritive value of barley protein in a properly supplemented diet. Therefore the amount of barley protein in all diets was kept at a constant level, which was determined by the 1952 barley, the lowest in protein.

TABLE 4

Swine feeding trial

(Animals fed individually for 84 days)

SUPPLEMENTAL PROTEIN SOURCE	1952 BARLEY [1]				1955 BARLEY [1]			
	Initial wt.	Average daily gain	Average daily ration	Feed per 100 lbs. gain	Initial wt.	Average daily gain	Average daily ration	Feed per 100 lbs. gain
	lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*
Casein to raise total protein to 10%	44.7	1.36	4.18	305.9	42.8	1.46	4.47	307.2
Casein to raise total protein to 16%	43.8	1.64	4.92	301.0	42.2	1.73	5.49	317.4
Soybean meal-cottonseed meal (60/40) to raise total protein to 10%	43.3	1.08	3.84	354.8	42.3	1.00	3.66	367.4
Soybean meal-cottonseed meal (60/40) to raise total protein to 16%	43.3	1.47	4.54	308.2	42.3	1.61	4.94	307.0

[1] Three animals were fed each diet.

The barleys were studied at two levels of total protein on the assumption that small differences in the value of the barley proteins might not be noted in the presence of an excess of supplemental protein. The maximum level of 16% of protein was chosen since it has been shown that, in general, pigs will reach maximum gain near this level (Jensen et al., '55). The 10% level was chosen because the data from the rat experiments reported herein suggested that level as being most satisfactory in comparing the barley proteins. Casein was chosen as one supplemental protein in order to compare the data from both swine and rats. The soybean-cottonseed mixture was chosen as the other source of supplemental protein, because it is the basis of many practical rations.

An inspection of the data (table 4) did not reveal any difference between the 1955 barley which had been freshly harvested and the 1952 barley which had been stored for three years. An analysis of variance also indicated that there was no statically significant difference between weight gains obtained from stored and fresh barley when fed to pigs. It should be remembered that barley in this experiment was serving as a major portion of the protein: 73% in the case of the 10% protein ration and 55% in the 16% ration. Therefore, it must be concluded in this study that storage had no deleterious effect on the quality of barley protein.

The average daily gains and the feed efficiency values obtained compared favorably with those reported by other workers using well balanced diets (Jensen et al., '55; Becker et al., '54). The average daily gain of the pigs receiving casein was considerably higher in all cases than that of those receiving the plant protein. This was as expected, but it should be pointed out that at the 16% level the differences were much smaller than those at the 10% level. The values for average daily gain were analyzed by the method of analysis of variance (Snedecor, '46). In the overall analysis highly significant differences were indicated in the case of casein versus soybean-cottonseed and in the case of 10% of total protein versus 16%. When the diets containing 10% of protein were compared, the

difference between sources of protein was highly significant. At the 16% level the difference was significant.

Feed efficiency values certainly indicate that the animals were making good use of their feed. Even at a level of 10% of total protein in the case of those animals receiving casein, the feed efficiency was excellent. The animals supplemented with soybean-cottonseed meal at the 10% level required more feed to produce 100 pounds of gain than any of the others.

SUMMARY

Under the conditions of these studies the nutritive value of barley protein was not decreased when the grain was stored for as long as three years.

No significant differences were noted in the nitrogen retention or the weight gain of growing rats fed stored or fresh barley either as the only source of protein or as a partial source of protein.

In the case of growing-fattening swine, the average weight gains and feed efficiency values obtained from feeding barley which had been stored for three years were equal to those obtained with new-crop barley when both barleys were supplemented with equal amounts of casein or equal amounts of a soybean-cottonseed meal mixture.

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THE EFFECT OF WITHDRAWAL OF ESTROGENS ON THE NITROGEN, CALCIUM AND PHOSPHORUS BALANCES OF WOMEN [1,2]

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This report presents the nitrogen, calcium and phosphorus balances of three women on constant diets before and after the withdrawal of natural or synthetic estrogen. Studies of the effects of natural estrogenic compounds or of the synthetic compound, diethylstilbestrol, on nitrogen and mineral metabolism have generally been made in cases in which the therapy was being used in an effort to arrest osteoporotic changes (Reifenstein and Albright, '40, '47), to evoke sexual development (Knowlton et al., '42), to alleviate cancer symptoms (Schilling et al., '50), or to revitalize the tissues of elderly persons (Kountz, '51). In most cases, the individuals were ill or senile. The general conclusions from earlier studies have been that nitrogen retention due to estrogen therapy is transitory (Albright and Reifenstein, '50), and that the anabolic effect of estrogen may be confined to genital tissue

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(Kountz, '51). The protein-sparing action of estrogen in senile cases could be observed only when the protein in the diet was less than 1 gm per kilogram of body weight per day (Kountz et al., '55). Estrogen therapy decreased the calcium and phosphorus excretions in individuals with osteoporosis of varying types (Albright and Reifenstein, '50) and in one case of carcinoma of the prostate (Schilling et al., '50).

The purpose of this investigation was to determine whether or not there were measurable changes in the nitrogen, calcium and phosphorus balances of healthy women on withdrawal of estrogens. In addition to the nitrogen and mineral balances of the three subjects, urinary excretions of 17-ketosteroid, pregnanediol and corticoids were determined.

EXPERIMENTAL PROCEDURES

Three healthy women served as subjects.[5] Subject A and subject B each had had hysterectomy and bilateral salpingo-oophorectomy several years previously. After this surgery, these women had been taking regularly physiological amounts of diethylstilbestrol (0.25 to 0.50 mg per day). These subjects were studied first while continuing their usual diethylstilbestrol therapy. Then, the diethylstilbestrol was withdrawn and, after a period of a minimum of 45 days without the estrogen, these two subjects were observed for a second time. Subject C was studied preceding, and 60 days after, hysterectomy and bilateral salpingo-oophorectomy without diethylstilbestrol therapy.

For each subject, the periods of observation consisted of a 5-day preliminary period for adjustment to a constant weighed diet, followed immediately by two 5-day periods on the identical diet, when collections of excreta were made. These 15-day periods were spent in the metabolic ward of the hospital.

[5] These women were patients of Dr. M. Edward Davis of the Department of Obstetrics and Gynecology, Chicago Lying-in Hospital.

Case histories. Subject A (L.I.H. 517173) was a 40-year-old white female with no children. Since 1951 following hysterectomy and bilateral salpingo-oophorectomy, 0.5 mg of diethylstilbestrol had been taken orally 4 times a week. The physical examination upon entering the hospital in October 1953 for the metabolic study showed subject A to be in good health with data on pulse rate, blood pressure and blood constituents within the normal ranges for her age. Her weight was 46.3 kg and height, 157 cm. A basal metabolic rate of −7% indicated a basic caloric requirement of 1200 Cal. per 24 hours. Diethylstilbestrol therapy was discontinued for subject A when she left the hospital at the end of the first balance period. She re-entered the hospital 45 days later for the second balance period.

Subject B (L.I.H. 346800), a 54-year-old white widow with two grown children, entered the hospital for the first balance study on November 10, 1953. In 1949 she had had hysterectomy with bilateral salpingo-oophorectomy. She had been taking, since the operation, 0.25 mg of diethylstilbestrol daily, except for a 30-day period in December 1952. This dosage was increased to 0.5 mg daily for the 10 days preceding and during the first study period. Her weight was 65.7 kg and height, 168 cm. Physical examination showed subject B to be in good health with normal pulse rate, blood pressure and blood composition. A basal metabolic rate of −11% indicated a basal requirement of 1270 Cal. per 24 hours. Upon leaving the hospital following the first study period, subject B discontinued the estrogen therapy, and began to experience "hot flushes" 6 days later. She re-entered the hospital for the second part of the balance study 70 days later, on January 31, 1954.

Subject C (L.I.H. 400342), a 40-year-old white widow with one grown son, entered the hospital December 8, 1953 for the first balance study. She had had rheumatoid type arthritis for the past 15 years, and had been taking cortisone from June to December 1, 1953. Examination on entering the hospital showed her to be in good health. While the cortisone

may have influenced the balance of nitrogen and the two minerals studied, her physician did not feel that it would affect the results. Her blood pressure, pulse rate, and blood composition were normal. She weighed 60.2 kg and her height was 172 cm. A basal metabolic rate of —15% indicated a basic requirement of 1250 Cal. per 24 hours. Hysterectomy and bilateral salpingo-oophorectomy were performed on January 2, 1954. Recovery from the operation was rapid and uneventful. Subject C left the hospital on January 16; no therapy was prescribed. On March 1, 1954 she re-entered the hospital for the second balance study. She was experiencing "hot flushes," having as many as 20 each day.

Diets. Each individual received the same weighed diet from day to day during each of the observation periods, but the diets differed somewhat from individual to individual. The diets contained approximately 6 to 8 gm of nitrogen and 250 to 350 mg of calcium each 24 hours. At these levels of intake of nitrogen and of calcium, any effect of withdrawal of estrogen would be more apparent than at higher dietary levels of the two nutrients.

Amounts of other nutrients were within the range of normal requirements with the caloric intake calculated to maintain a constant body weight throughout each study period. Subject A's daily food intake was calculated to average 1400 Cal., 6.96 gm of nitrogen, 351 mg of calcium, 690 mg of phosphorus, 11 mg of iron, 4900 I.U. of vitamin A, 0.41 mg of thiamine per 1000 Cal., 0.84 mg of riboflavin, 10.32 mg of niacin and 80 mg of ascorbic acid. Subject B's daily food intake was calculated to average 1740 Cal., 7.46 gm of nitrogen, 341.5 mg of calcium, 705 mg of phosphorus, 11 mg of iron, 5700 I.U. of vitamin A, 0.60 mg of thiamine per 1000 Cal., 0.92 mg of riboflavin, 14.14 mg of niacin and 126 mg of ascorbic acid. Subject C's daily food intake was calculated to average 1670 Cal., 7.24 gm of nitrogen, 392 mg of calcium, 712 mg of phosphorus, 11.13 mg of iron, 3940 I.U. of vitamin A, 0.52 mg of thiamine per 1000 Cal., 0.96 mg of riboflavin, 11.6 mg of niacin, and 180 mg of ascorbic acid.

Thus the diets were planned so that the foods consumed by the subjects would contain the essential nutrients in adequate quantities, in their natural form and in balanced proportions. It was believed that in following this plan, normal diets could be weighed and served the subjects, diets which were acceptable and which eliminated the necessity of mineral supplements. Because the milk in the diets was low, in order to keep the calcium levels low, it was necessary to add synthetic riboflavin to each diet. A 2 mg supplement of riboflavin was taken with each evening meal.

Clinical and chemical procedures. At the beginning of each 15-day period of observation, data for each subject included basal metabolic rate, blood pressure, pulse rate, and blood constituents (hemoglobin, red and white cell counts, cell volume, serum protein, N.P.N., Na, K), as well as roentgenograms of the lumbosacral spine, pelvis, and right femur and knee.

The standard techniques [6] for the conduct of nitrogen, calcium, and phosphorus balances were carried out. Nitrogen in homogenized food and fecal composites and in urine was determined by a semi-micro Kjeldahl method as developed by Cole and Parks ('46). Calcium was determined by the McCrudden method ('11–'12) and phosphorus by the method of Fiske and Subarrow ('25) in the same composites. Urinary 17-ketosteroids were determined [7] by the method of Holtorff and Koch ('40), pregnanediol by a modified method of Sommerville, Marrian and Kellar ('48), and corticoids by a modified method of Corcoran and Page ('48).

RESULTS

There were no significant changes in body weight among the subjects during the balance periods. Subject A maintained

[6] Details of the standard techniques for nitrogen and mineral balances as carried out in this investigation appear in a thesis by Eloise S. Cofer entitled, "The Effect of Withdrawal of Estrogens on the Nitrogen, Calcium and Phosphorus Metabolism of Three Women." March 1955, available from the University of Chicago library.

[7] These determinations were done in the Endocrinology Laboratory of Chicago Lying-in Hospital.

a weight of 46.3 kg throughout the study; subject B weighed 65.7 kg in period I and 66.4 during period II; subject C's weight was 60.2 kg in period I and 59.8 in period II. Subject A's diet averaged 30 Cal. per kilogram of body weight; those of subjects B and C averaged 27.5 and 27.6 Cal. per kilogram of body weight, respectively.

The daily protein intake provided 0.91 gm per kilogram for subject A in period I and 1.0 gm per kilogram of body weight in period II. For each of the study periods subject B's diet provided 0.76 gm of protein per kilogram of body weight while subject C's diet provided 0.82 gm of protein. These intakes of protein represented daily nitrogen consumptions which ranged from 6.76 to 8.12 gm, figures very close to the 6 to 8 gm allowance originally planned for the weighed diets.

Table 1 shows the average daily intakes, excretions and resulting positive or negative balances of subjects A, B and C for the 10-day observation on weighed diets. The 10-day period while estrogen was being administered at the level of 0.5 mg of diethylstilbestrol per day, or was being naturally secreted, has been designated as period I; the 10-day period after withdrawal of diethylstilbestrol or after oophorectomy, as period II. Data for the 5-day preliminary adjustment periods are not shown.

Nitrogen balances were slightly positive and ranged from $\pm$ 0.46 to $\pm$ 1.16 gm of nitrogen per day during all periods for subjects A and C. Subject B was also in positive nitrogen balance ($\pm$ 0.47 gm N) in period I with diethylstilbestrol, but she was in slight negative nitrogen balance (— 0.50 gm N) after diethylstilbestrol was withdrawn. Examination of the data in table 1 makes it apparent that the small decreases in retention of nitrogen for each subject in period II were a reflection of increases in urinary excretions and were not a result of significant changes in fecal nitrogen excretion. In comparing the urinary excretions of nitrogen with nitrogen intakes, it was found that, for subject A, there was no change in the percentage of dietary nitrogen appearing in the urine

TABLE 1

Average 10-day nitrogen, calcium and phosphorus balances of three women with and without estrogens

SUBJECT AND PERIOD	DAYS	TREATMENT	NITROGEN				CALCIUM				PHOSPHORUS			
			Intake	Output		Balance	Intake	Output		Balance	Intake	Output		Balance
				Urine	Feces			Urine	Feces			Urine	Feces	
			gm/24 hrs.				*mg/24 hrs.*				*mg/24 hrs.*			
Subj. A														
Per. I	10	0.5 mg/day diethylstilbestrol	6.76	5.36	0.80	+0.60	258	153	292	—187	527	413	196	—82
Per. II	10	None	7.39	6.06	0.87	+0.46	299	233	309	—243	606	555	232	—181
Subj. B														
Per. I	10	0.5 mg/day diethylstilbestrol	8.07	6.40	1.20	+0.47	301	25	266	+10	680	412	285	—17
Per. II	10	None	8.12	7.54	1.07	—0.50	391	60	392	—61	798	505	280	+13
Subj. C														
Per. I	10	Pre-operative (Corpus luteum phase)	7.92	5.53	1.22	+1.16	347	164	293	—110	596	490	225	—119
Per. II	10	Post-operative	7.72	6.05	1.18	+0.49	345	211	360	—226	644	501	237	—94

on withdrawal of the estrogen, the urinary nitrogen averaging about 80% of the nitrogen intake during each of the two metabolic periods. For subject B in period I, nitrogen excretion in the urine was 80% of the intake, and for period II it averaged 92% of the intake. For subject C, urinary excretion of nitrogen was 70 and 78% of the intake for the pre-operative (corpus luteum phase) and post-operative periods, respectively.

The calcium balance, which was negative to the extent of — 187 mg per 24 hours in the treatment phase for subject A, changed to an average of — 243 mg of calcium per 24 hours for the period after withdrawal of the estrogen. For subject B, with an initial positive balance of + 10 mg of calcium per day in period I, a negative calcium balance of — 61 mg was recorded during period II. Subject C more than doubled the negativity of her calcium balance in the post-operative as compared with the pre-operative metabolic period, the average calcium balance in period I being — 110 mg per day, in period II, — 226 mg per day.

From a study of the data in table 1, it appears that the urinary calcium excretion was an important factor in the increased negative calcium balances for subjects A and B in the periods after withdrawal of estrogen. For subject A, the average urinary calcium increased from 153 mg per 24 hours during diethylstilbestrol treatment (period I) to 233 mg per 24 hours in the study periods after withdrawal of estrogen. The urinary calcium accounted for 34% of the total excretion in period I and 43% in period II. The increase in fecal calcium excretion between the two periods paralleled closely the small increase in calcium intake in period II. For subject B, the urinary calcium was approximately 9% of the calcium excretion in period I and 13% in period II. While the fecal calcium excretion in period I was 35 mg per day less than the dietary intake of calcium, in period II it was 2.1 gm more than the intake. From these observations it is apparent that the percentage of urinary calcium excretion in relation to the total calcium output is an important factor

in the increased negative balance for subjects A and B in the period after withdrawal of estrogen. Urinary calcium excretion for subject C represented about the same percentage of total excretion in each period, and further, the fecal and urinary calcium increases in the post-operative period played equally important roles in producing an increased negative balance.

The phosphorus balances showed no consistent trend from period I to period II for the three subjects. Subject A was in greater negative phosphorus balance in period II (— 181 mg per day) than in period I (— 82 mg per day). Subject B, on the other hand, shifted from a negative phosphorus balance of — 17 mg per day in period I to + 13 mg per day in period II, both balances being very close to equilibrium. Subject C, also, had less loss of phosphorus in period II, showing a 119 mg loss each day in period I and a 94 mg loss each day post-operatively. For each of the subjects, the urinary excretions of phosphorus in period II exceeded those of period I. Subjects B and C maintained about the same urinary phosphorus excretion in relation to intake for each period. However, for subject A the urinary phosphorus rose from 78% of the intake in period I to 91% in period II.

The urinary 17-ketosteroid, pregnanediol, and corticoid values are recorded in table II. The 17-ketosteroid excretions for subject A ranged from 5.8 to 9.1 mg per 24-hour period; for subject B, these determinations ranged from 12.4 to 15.9 mg per 24-hour period. Pregnanediol values in the urine for subject A ranged from 1.43 to 4.35 mg per 24 hours in period I and from 3.46 to 5.20 mg per 24 hours in the period without diethylstilbestrol. For subject B, the pregnanediol determinations fluctuated between a low of 0.41 mg to a high of 3.04 mg per 24 hours in period I. In period II, without diethylstilbestrol, the values ranged from 2.11 to 2.82 mg per 24 hours. Subject C's pregnanediol excretions during the pre-operative study-period (corpus luteum phase) were variable, 8.20, 2.46, and 3.01 mg per 24 hours for one three-day and two two-day urine composites in that order.

TABLE 2

The 17-ketotseroid, pregnanediol, and corticoid values for women with and without estrogen

SUBJECT	PERIOD	DATE	17-KETO-STEROID	PREGNANE-DIOL	CORTI-COIDS
			mg/24 hrs.	*mg/24 hrs.*	*mg/24 hrs.*
A	I	9/29/53	6.48	1.43	0.81
		9/30/53	6.48	1.43	0.81
		10/1/53	5.80	...	...
		10/2/53	5.80	...	...
		10/3/53	6.30	...	...
	Estrogen	10/4/53	6.30	...	...
	treatment	10/6/53	6.80	...	...
		10/7/53	6.80	...	...
		10/9/53	6.90	...	...
		10/10/53	6.90	...	...
		10/11/53	...	4.35	1.02
		10/12/53	...	4.35	1.02
	II	11/28/53	6.90	5.20	1.36
		11/29/53	6.90	5.20	1.36
		11/30/53	6.90	5.20	1.36
	No	12/3/53	6.90	3.46	...
	treatment	12/4/53	6.90	3.46	...
		12/5/53	9.10	4.25	1.25
		12/6/53	9.10	4.25	1.25
		12/7/53	9.10	4.25	1.25
B	I	11/10/53	13.00	3.04	0.52
		11/11/53	13.00	3.04	0.52
		11/12/53	13.00	3.04	0.52
		11/13/53	12.60	0.79	0.61
		11/14/53	12.60	0.79	0.61
	Estrogen	11/15/53	12.60	0.79	0.61
	treatment	11/16/53	12.40	1.08	0.43
		11/17/53	12.40	1.08	0.43
		11/19/53	12.40	1.08	0.43
		11/20/53	14.00	0.41	0.76
		11/21/53	14.00	0.41	0.76
		11/23/53	14.00	0.41	0.76
	II	2/8/54	15.80	2.11	1.02
		2/9/54	15.80	2.11	1.02
	No	2/10/54	15.80	2.20	...
	treatment	2/11/54	15.80	2.20	...
		2/12/54	15.90	2.82	0.96
		2/13/54	15.90	2.82	0.96
		2/14/54	15.90	2.82	0.96
C	I	12/16/53	9.10	8.20	1.32
		12/17/53	9.10	8.20	1.32
		12/18/53	9.10	8.20	1.32
		12/19/53	13.90	...	...
		12/20/53	13.90	...	...
	Pre-	12/21/53	13.90	...	...
	operative	12/22/53	11.20	2.46	...
		12/23/53	11.20	...	...
		12/24/53	7.90	...	...
		12/25/53	7.70	...	...
		12/26/53	14.70	3.01	...
		12/27/53	14.70	3.01	...
		12/28/53	19.70	...	1.10
		12/29/53	19.70	...	1.10
		12/30/53	19.70	...	1.10

The values for corticoids for subjects A and B were low in period I while the subjects were taking diethylstilbestrol, falling below the 1.0 to 1.5 mg per 24 hours considered normal. The values were higher in the periods after withdrawal of the estrogen. For subject A the values for period I ranged from 0.81 to 1.02 mg per 24 hours. In period II, the values were 1.36 and 1.25 mg per 24 hours for two three-day urine composites. For subject B, the values were from 0.43 to 0.76 mg per 24 hours, during period I. In period II, after withdrawal of the diethylstilbestrol, the values were slightly higher, from 0.96 to 1.02 mg per 24 hours.

Clinical data remained normal throughout the periods of observation of these subjects and roentgenograms showed no changes in osseous structures.

DISCUSSION

The three women who served as subjects in this study remained in good health during the 15-day periods of observation, were adequately supplied with nutritional requirements by the experimental diets and maintained constant body weights. The similarity of kinds and amounts of food in the diet of each subject resulted in an intake with about the same distribution of calories from protein, fat, and carbohydrate. The relatively high levels of fat in the diets (34 to 43%) might be considered to have had an effect on calcium absorption. Since the percentage of calories from fat deviated little from period to period for each subject, calcium absorption should have been equally influenced throughout the period of observation by this dietary factor, if at all.

Nitrogen balances. The nitrogen balances for the three subjects were close to equilibrium on low protein intakes providing approximately 6 to 8 gm of nitrogen per day. In period I, when subjects A and B were on diethylstilbestrol, as they had been for several years, and when subject C was in the corpus luteum phase of the estrous cycle, all of the nitrogen balances were positive. In other words, some nitrogen was retained from the restricted intakes of protein when

estrogens, either synthetic or natural, were available. On the other hand, while the nitrogen balances in two cases in three remained slightly positive on nearly the same intakes of nitrogen, there was a reduced nitrogen retention (an increased nitrogen excretion) when the effect of estrogens was removed either by withdrawal of diethylstilbestrol or by surgical extirpation of tissues supplying natural estrogens. Although Albright and Reifenstein ('50) noted a "poorly sustained" effect of estrogen for improving nitrogen retention, the data in this study indicate a consistent trend toward increased nitrogen retention from a constant restricted dietary supply when estrogenic effects were present. The ability of the subjects in this study to maintain a positive nitrogen balance on the low levels of protein intake was better than that of older subjects for whom Kountz reported ('55).

Calcium and phosphorus balances. Although subject B normally consumed somewhat larger quantities of calcium from milk and cheese, the experimental diets provided calcium in a quantity typical of the usual diets of subjects A and C. It was considered important to restrict the calcium intakes to levels of from approximately 250 to 350 mg so that they would be sensitive to possible estrogenic influences. Similar levels of calcium intake were employed by Knowlton and others ('42) in their studies which compared estrogen and testosterone therapy. The calcium balances of subjects A and C were negative during period I; that of subject B was slightly positive, showing a retention of 10 mg per day. In period II, when estrogens were withdrawn, all three subjects showed increased urinary calcium excretion and negative balances. This trend toward loss of calcium when the effect of estrogen was removed was indicated also in studies of the effect of estrogen therapy on calcium balance by Reifenstein and Albright ('40, '47), Schilling et al. ('50), and others.

The failure to note a consistent trend in the phosphorus metabolism of these subjects, as measured by phosphorus balances during constant diets, can not be explained. Only

for subject A was the response in the expected direction in the light of the changes in the nitrogen and calcium balances with and without estrogens. Phosphorus excretions were greater in period II without diethylstilbestrol than in period I, for subject A. For subjects B and C the phosphorus excretions were lower in the absence of estrogenic effects.

Urinary constituents. There were some increases in the urinary 17-ketosteroid values after the removal of diethylstilbestrol from subjects A and B, but there was not sufficient change between the levels of excretion of these compounds in the two periods to warrant the assumption that it was due to the withdrawal of estrogen. Similarly there was no change in pregnanediol excretion which seemed significantly related to the presence or absence of estrogen therapy.

The depression of the urinary corticoid values for subjects A and B during the period with diethylstilbestrol and the increase in the excretion values upon withdrawal of the estrogen may be worthy of note. These data are at variance with the findings of Zondek and Burstein ('52), who report an elevation of corticoid values in the urine of spayed guinea pigs given a 500 mg dose of diethylstilbestrol.

SUMMARY AND CONCLUSIONS

1. The effect of withdrawal of diethylstilbestrol therapy in two oophorectomized subjects and of oophorectomy in one subject on the nitrogen, calcium, phosphorus balances and on the urinary 17-ketosteroid, pregnanediol, and corticoid excretions was investigated.

2. For each subject there was a slight increase in nitrogen excretion upon withdrawal either of diethylstilbestrol or of natural estrogens. Two of the subjects exhibited excretions increased to an extent to justify the conclusion that in these subjects at a given level of nitrogen intake, withdrawal of estrogenic influences may have been responsible for the increased nitrogen loss.

3. With calcium intakes of 250 to 350 mg per day, the three subjects showed negative calcium balances that were significantly increased after withdrawal of estrogens. It appears that cessation of the estrogenic effect resulted in an increased calcium output, urinary excretion being the chief pathway for the loss of the mineral in one subject (A) and urinary and fecal excretion being jointly responsible in subjects B and C.

4. For one subject (A), the phosphorus balances during the two periods of study paralleled the calcium balances, becoming increasingly negative in the period without diethylstilbestrol. The phosphorus balances of the other two subjects did not follow this direction.

5. The 17-ketosteroid and pregnanediol values were in the normal range for all periods studied. Subjects A and B appeared to have depressed urinary corticoid values while on diethylstilbestrol therapy.

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TRANSFER OF PHOSPHATE IN THE DIGESTIVE TRACT

IV. TURKEYS [1]

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The transfer of intravenously injected radiophosphate into the digestive tract of various domestic mammals has been previously reported (swine, Smith et al., '55a; sheep, Smith et al., '55b; and dairy cattle, Smith et al., '56b). The digestive tract of birds is different from that of mammals: a storage organ, the crop, precedes the acid-secreting stomach, the proventriculus, and a macerating organ, the gizzard or ventriculus, follows it. The crop of the bird differs from the rumen of some mammals in that it is rapidly evacuated, and it is not the site of any appreciable digestion. The gizzard functionally replaces the teeth of mammals.

The intestines of the bird are short as compared with those of mammals of similar size, and are relatively undifferentiated. Two large ceca are attached to the intestine at the junction of the ileum and colon. The colon and ureters both empty into the cloaca, from which liquid urine enters the colon where it is dehydrated and later voided as a white paste on the feces.

There is, however, a general similarity of function in mammalian and avian digestive tracts. Similar digestive juices are found in each, although with some variations in their sites of formation, contents of enzymes, pH, etc. Detailed descriptions of the structure and function of the avian diges-

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tive tract can be found in the recent books by Bradley and Grahame ('50), Calhoun ('54), and Sturkie ('54). The microanatomy of the duodenum of the turkey, which is somewhat different from that of the chicken, has been described by Rosenberg ('41).

There also appear to be quantitative differences in the mineral requirements of domestic birds and mammals. The phosphorus requirement (per kilogram of body weight) is about 5 times as great for turkeys (excluding requirement for egg production) as for sheep and dairy cattle, and double that for swine (Albritton, '53).

These nutritional and functional differences between avian and mammalian digestive tracts might result in differences in the transfer of phosphate therein. In the experiments described herein, radiophosphorus (P^{32}) has been used to label the circulating phosphate of turkeys. The appearance of this material in the gastrointestinal contents has been used to evaluate the transfer of phosphate into the digestive tract.

METHOD

Mature Broad-Breasted Bronze turkeys, all about 16 months old, were taken from the departmental flock for this experiment. Three were females, two of which were in egg production, with a mean body weight of 7.8 ± 0.6 kg; and three were males, with a mean body weight of 13.2 ± 0.8 kg. These animals and the radiophosphate dosage have been previously described (Smith et al., '56a).

High environmental temperatures, such as existed outdoors at the time of these experiments, cause considerable increases in water consumption and fluidity of the droppings (Wilson, '49). Since this condition might affect the secretion and movement of phosphate in the digestive tract, the birds were kept in cages in a room maintained at 65°F. for a week prior to and during the experiment.

Each bird was injected via a wing vein with a buffered isotonic solution containing 0.6 to 1.4 mc of labeled phosphate.

At times varying from one to 24 hours after the injection, the birds were sacrificed by exsanguination. Samples of contents were taken from the crop, proventriculus, gizzard (freed of grit), duodenum (posterior to the entry of bile and pancreatic juice), ileum, cecum, and colon. The excreta of the birds sacrificed at 24 hours after the injection were collected over the period of the experiment. Most birds had a slight infestation of roundworms in the duodenum, a few of which were collected. Samples of bile were withdrawn from the gall bladder with a syringe and hypodermic needle.

The various samples were dried at 105°C., treated with magnesium nitrate (1 ml of a 10% $Mg(NO_3)_2$ solution per gram of dry matter), redried, and ashed at 500°C. The ash was taken up in dilute hydrochloric acid.

A pre-mortem blood sample was drawn from each bird, from which was prepared a deproteinized filtrate of plasma (1 vol. plasma treated with 4 vols. of 10% trichloroacetic acid, and filtered after extraction for 20 mins).

Aliquots of all the prepared solutions were dried on copper planchets, and their radioactivity was determined with a Geiger-Müller counter. The phosphorus concentration of these solutions was also determined by the method of Koenig and Johnson ('42).

RESULTS AND DISCUSSION

The results of the chemical analyses (dry matter and phosphorus content of the dry matter) are presented in table 1 as the means and their standard errors. The dry matter content of the digesta was quite variable in the anterior part of the tract. In the crop, it varied from 10% in one animal in which the content was mainly mucous, to 75% in others in which the crop was fully packed with feed. In all birds the content of the proventriculus was limited to a scanty mucous secretion — in most cases too little for a useful sample. Generally the dry matter content of the turkey digesta was greater than previously found in mammals, except

in the colon, where the situation was the reverse. This smaller amount of dry matter in the colon contents may result from the entry of liquid urine from the cloaca.

The phosphorus content of the digesta anterior to the small intestine is comparatively quite low. Material in the gizzard has a lower phosphorus concentration than that of the crop, indicating that digestion in this organ is accompanied by an extraction of phosphorous compounds. In the duodenum, the

TABLE 1

Dry matter and phosphorus content of turkey digesta

SEGMENT OF DIGESTIVE TRACT	DRY MATTER	PHOSPHORUS CONTENT
	%	*mg P/gm dry*
Crop	30.6 ± 11.8	6.9 ± 0.2
Proventriculus	24.9 ± 2.7	2.2 ± 0.5
Gizzard	32.1 ± 1.6	3.3 ± 0.7
Duodenum	15.0 ± 1.6	12.5 ± 0.5
Ileum	16.5 ± 1.4	12.9 ± 0.4
Cecum	18.8 ± 2.2	24.6 ± 2.3
Colon	14.0 ± 2.5	18.4 ± 2.0
Worms	27.5 ± 2.8	7.2 ± 0.8
Bile	15.0 [1]	0.59 ± 0.14 [2]
Excreta	...	6.2 ± 0.5

[1] Single determination.

[2] Milligrams phosphorus per milliliter gall bladder bile; 41.3 mg P/gm dry matter on the basis of the single dry matter determination. This may not be characteristic of all turkey bile, since one lobe drains to the duodenum apart from the gall bladder.

phosphorus content of the digesta is quite high, much higher than in the duodenal digesta of swine, sheep, or dairy cattle, and it increases lower down. The phosphorus content of cecal digesta is even higher, being double the value of the ileac digesta, indicating either a secretion of phosphorus or a preferential absorption of non-phosphorous materials in this organ. The phosphorus content of the colon digesta is intermediate between that of ileum and cecum. This increased phosphorus in the colonic contents is probably from urine entering this organ, rather than from the cecal contents, since

the latter are voided periodically and appear distinctly separate in the droppings. Most of the phosphorus in the colonic digesta must be resorbed prior to their excretion, since the phosphorus content of the droppings is much lower.

The phosphorus and radioactivity analyses of the digesta were used to calculate their specific activities (microcuries P^{32} per milligram of phosphorus). These figures, standardized for dosage (millicuries P^{32} per kilogram of body weight), are presented in table 2. The specific activities of the contents

TABLE 2

Standard specific activities of contents of the gastrointestinal tracts of turkeys

	μc P^{32}/mg P					
	mc P^{32} inj./kg body weight					
EXPERIMENT NO.	T1	T2	T3	T4	T5	T6
HOURS, POST-INJECTION	1	3	6	9	24	24
SEX	♀	♂	♀	♂	♀	♂
Plasma inorganic P	5.20	5.40	1.43	2.20	0.58	0.93
Crop	...	0.05	0.02	0.01	0.03	...
Proventriculus	0.39	0.40	...	...	...	...
Gizzard	0.02	0.08	0.13	0.01	0.01	0.03
Duodenum	1.49	1.25	0.45 [1]	0.33	0.10	0.06
Ileum	0.37	2.29	3.00	1.29	0.42	0.08
Cecum	...	1.39	2.76	1.21	0.60	1.47
Colon	1.14	1.85	8.10	1.44	0.02	...
Worms (duodenal)	...	0.12	0.04	...	0.04	0.09
Bile	0.40	1.38	0.41	1.72	1.08	2.82

[1] Upper duodenum (pre-bile), 0.29.

of the crop, proventriculus, and gizzard are quite low at all times after injection of the tracer, indicating only a small secretion of endogenous phosphorus in these organs.

One hour after administration of the tracer, the highest specific activity was observed in the digesta of the duodenum, indicating it to be the main site of phosphorus secretion. At this time, the duodenal contents have a much higher specific activity than does bile, indicating that most of the secreted phosphorus (probably phosphate) is from some other source (pancreatic juice or succus entericus). The specific activity of

the bile, however, continues to increase, probably as the result of labeling slower-forming organic phosphorous compounds, until it is much higher than the plasma inorganic phosphorus 24 hours after injection. There is a difference between the two sexes ($\delta > \varphi$) in the rate of increase in bile specific activity.

Beyond one hour after tracer injection, the specific activity of the duodenal contents decreases; this results from a decrease in the specific activity of circulating phosphate as well as from dilution with (unlabeled) food phosphorus. At these later times, the specific activity of the contents posterior to the duodenum is much higher, partly because of the movement of material along the digestive tract. Hillerman et al. ('53) reported the average time for passage of feed through the digestive tract of the laying turkey hen to be about three hours, and for the non-layer about 4 hours.

The specific activity of the colonic contents, which was second to the duodenum one hour after injection, increased rapidly to a maximum at 6 hours. This appearance of labeled phosphorus in the colon probably resulted from the entry of urine into the lower intestine rather than from a secretion by the intestine. This extremely high deposition of endogenous phosphorus 6 hours after injection and the lack of it at 24 hours after injection are probably associated with shell formation. Common ('46) observed a surging of phosphorus excretion accompanying shell formation. This extra phosphorus is readily soluble in water and is associated with the urinary portion of the droppings, whereas phosphorus in droppings produced between periods of shell formation is less water-soluble and is associated with the fecal portion. This periodic excretion of phosphorus results from a similar fluctuation in the level of blood phosphorus, which has been described by Feinberg et al. ('37). When sacrificed, turkey T3 (6 hrs.) had a hardshelled egg in the uterus and, accordingly, would have been approaching the end of the period of augmented phosphorus excretion; turkey T5 (24 hrs.) had laid an egg

6–8 hours before sacrifice and would thus have been in a period of minimal phosphorus excretion.

The relatively lower specific activity of the plasma inorganic phosphorus and bile phosphorus in the female probably result primarily from a more rapid turnover of phosphorus rather than from the variable level of blood inorganic phosphorus. During the experiment, turkey T5 (♀, 24 hours) excreted 17% of the administered tracer dose (in egg and droppings) whereas turkey T6 (♂, 24 hours) excreted only 3% of the tracer dose (droppings only).

The cecal digesta had a lower specific activity than those of either ileum or colon, except at 24 hours after injection. This would indicate only a small secretion of endogenous phosphorus in this organ; consequently, the high phosphorus concentration in cecal digesta, previously noted, probably results from a relatively greater resorption of non-phosphorous compounds. The higher specific activity of cecal contents 24 hours after injection may result from a slow rate of exchange of this material with intestinal contents. In the chickens, the discharges of the colon may be as much as 11 times more frequent than cecal discharges (Sturkie, '54).

The duodenal roundworms that were collected from some birds had a low specific phosphorus activity throughout the experiment; only in one 24-hour bird did the specific activity of the worms exceed that of the duodenal contents, and it was much lower than that of the plasma inorganic phosphorus at all times. This would indicate that intestinal parasites exchange phosphorus only slowly with either blood or digesta.

SUMMARY AND CONCLUSIONS

The secretion of endogenous phosphorus into the digestive tract of turkeys was studied following intravenous injection of labeled phosphate (P^{32}). The duodenum receives most of the secretion, as is the case with mammals having a simple stomach (e.g., swine). Very little phosphorus secretion was indicated in the ceca, although their contents have the highest

phosphorus concentration of any part of the digestive tract. Appearance of endogenous phosphorus in the colon is associated with entry of urine into that organ.

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THE EFFECT OF 2-AMINO-2-METHYL-1-PROPANOL ON THE INCIDENCE OF KIDNEY LESIONS IN MALE RATS OF DIFFERENT AGES FED DIETS LOW IN CHOLINE[1]

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Renal lesions of choline deficiency have seldom developed in mature rats. Griffith ('40) showed that 25 to 30% of male rats over 30 days of age on low-choline diets developed hemorrhagic kidneys in 10 days. When male rats less than 30 days of age were fed low-choline diets, 75 to 100% developed hemorrhagic kidneys in 10 days. The hemorrhagic state in the latter group was so severe that several of the animals died. In the former group the kidney hemorrhages were moderate rather than severe. Death in this group did not occur. Griffith and Wade ('39) showed that the liver fat in animals over 35 days of age on diets low in choline was only one-half that in rats less than 30 days of age. Handler ('46) found that 250-gm rats on low-choline diets containing 15% of casein did not develop kidney lesions.

In a preliminary communication ('56), we reported the effect of 2-amino-2-methyl-1-propanol on the incidence of kidney lesions in male rats between three and 20 weeks of age. Wells ('55a) had reported that both 2-amino-2-methyl-1-propanol and α, α-dimethyltriethylcholine markedly increased the severity of choline deficiency in young rats. Both Wells

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('55b) and Mulford ('55) reported that betaine, methionine, and casein had little effect in overcoming the anticholine action of this drug.

The present report shows that male rats started on experiment when older than three weeks developed markedly severe kidney lesions when fed a low-choline diet containing 2-amino-2-methyl-1-propanol, while animals on the same diet without added 2-amino-2-methyl-1-propanol developed no kidney lesions.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain, three to 20 weeks of age, and on a commercial stock diet [2] were placed in raised cages and fed the experimental diets ad libitum. At intervals the rats were sacrificed by decapitation and the kidneys examined for hemorrhages.

The basel low-choline diet (TC-2) was similar to that of Mulford and Griffith ('42) and consisted of casein [3] 18, dry brewers' yeast [4] 6, agar 2, salt mixture [5] 4, cane sugar 48.7, lard 19.9, fortified fish liver oil [6] 0.1, calcium carbonate 1, and L-cystine 0.3%. Water was available to the animals at all times.

2-Amino-2-methyl-1-propanol in rats of different ages. The effect of the addition of 2-amino-2-methyl-1-propanol (2A2MIP) at levels of 10 and 20 mg per gram of diet TC-2 on the severity of kidney lesions in male rats of different ages is shown in table 1. The condition of the kidneys is recorded in the table as normal, slight, moderate, and severe.

Table 1 shows that the time when kidney lesions appeared as well as the time when the lesions were severe depended upon the age of the animals. In the three-week-old group kidney hemorrhages due to 10 mg of 2A2MIP appeared on about the 4th day and were severe in all animals on the 6th day. In

[2] Purina Laboratory Chow.
[3] General Biochemicals, Inc., vitamin free.
[4] Anheuser-Busch, Inc., strain G.
[5] General Biochemicals, Inc., Salt mixture no. XIV.
[6] Natola, Parke-Davis and Company.

the 5-week-old group kidney hemorrhages did not occur until the 7th day, and were not severe in all animals until the 9th day. In the 8-week-old group the lesions appeared on the 9th or 10th day and were severe in all rats on the 12th day. Kidney lesions were severe in all the 10-week and 11-week old animals after consuming diet TC-2 containing 20 mg of the drug per

TABLE 1

The effect of age on the severity of kidney hemorrhagic degeneration in male rats receiving low-choline diet TC-2, supplemented with 2-amino-2-methyl-1-propanol (2A2M1P)

2A2M1P PER GRAM FOOD	AGE OF ANIMALS	NO. OF RATS	DAY SACRIFICED	SEVERITY OF KIDNEY HEMORRHAGES				NO. OF DEAD	NO. RATS WITH OCULAR HEMORRHAGES
				Normal	Slight	Moderate	Severe		
mg	*weeks*			*number of animals*					
10	3	5	4	4	1	0	0	0	0
		5	5	0	0	1	4	0	0
		26	6	0	0	0	26	5	5
	4	15	6	7	4	2	2	0	0
		5	7	0	0	0	5	0	0
		5	8	0	0	0	5	1	0
	5	15	6	15	0	0	0	0	0
		5	7	0	0	3	2	0	0
		5	8	0	0	1	4	0	1
		5	9	0	0	0	5	2	1
	6	9	8	7	1	1	0	0	0
		3	9	1	0	0	2	0	0
		3	10	0	0	0	3	0	3
	7	3	9	1	1	1	0	0	0
		3	10	0	1	0	2	0	0
		4	11	1	0	1	2	0	0
		5	12	0	0	1	4	1	0
	8	3	10	1	1	0	1	0	0
		3	11	0	0	1	2	0	0
		9	12	0	0	0	9	0	0
20	10	6	9	0	0	5	1	0	0
		3	10	0	0	2	1	0	0
		2	12	0	0	0	2	0	0
		4	13	0	0	0	4	1	0
	11	4	9	0	2	2	0	0	0
		3	10	0	0	2	1	0	0
		2	11	0	0	1	1	0	0
		6	13	0	0	0	6	1	0

gram of food for 12 to 13 days. From the 9th to the 11th day the kidneys in these groups were moderately hemorrhagic.

Death as well as ocular hemorrhages (Griffith and Wade, '39) occurred at the time when most of the animals had severe kidney hemorrhages. In many rats showing the severe condition the kidneys were extremely large and out of shape. Oftentimes blood covered the back of the abdominal cavity. Before being sacrifieced many of the animals shook continuously. Many either voided a bloody urine or were unable to void urine at all.

TABLE 2

The effect of the dietary level of 2-amino-2-methyl-1-propanol (2A2M1P) on the severity of kidney hemorrhagic degeneration in male rats receiving low-choline diet TC-2

AGE	NO. OF RATS	2A2M1P PER GRAM OF FOOD	DAY SACRIFICED	INCIDENCE OF KIDNEY HEMORRHAGES	AVERAGE WEIGHT INCREASE PER RAT PER DAY	AVERAGE FOOD INTAKE PER RAT PER DAY
weeks		*mg*		*%*	*gm*	*gm*
9	6	10	11	17	1.6	12.8
9	6	20	11	100(1) [1]	— 1.9	9.9
10	15	10	12	27	1.9	15.9
10	15	20	12	93(2)	— 1.3	13.3
12	16	10	13	13	2.2	15.9
12	15	20	13	87(2)	— 2.6	13.3

[1] Numerals in parentheses represent the number of animals that died.

Effect of level of 2-amino-2-methyl-1-propanol on severity of kidney lesions. The effect of feeding the low-choline diet (TC-2) containing different levels of 2A2MIP is shown in table 2. Male rats 9, 10, and 12 weeks of age were divided into two groups. One group at each age received diet TC-2 plus 10 mg of drug per gram of food and the other, the same diet plus 20 mg of drug per gram of food. The body weight and food intake were recorded each day. Table 2 shows the adverse effect of the higher level of 2A2MIP. From 87 to 100% of the animals receiving this level of the drug had kidney lesions and each lost from 1.3 to 2.6 gm body weight per day. Only from 13 to 27% of the animals receiving the lower level

of the drug had kidney lesions and each gained from 1.6 to 2.2 gm in body weight per day. Food consumption for the 10-mg group ranged from 12.8 to 15.9 and, for the 20-mg group, from 9.9 to 13.3 gm per rat per day. Five animals died in the group receiving the higher level of the drug, while none died in the group receiving the lower level.

TABLE 3

The effect of choline on the incidence of kidney lesions in older male rats receiving low-choline diet TC-2 containing 2-amino-2-methyl-1-propanol (2A2M1P)

AGE OF ANIMALS	NO. OF RATS	SUPPLEMENTS ADDED PER GRAM OF FOOD		DAY SACRIFICED	INCIDENCE OF HEMORRHAGIC KIDNEYS	AVERAGE WEIGHT INCREASE PER RAT PER DAY	AVERAGE FOOD INTAKE PER RAT PER DAY
		2A2M1P	Choline Cl.				
weeks		*mg*	*mg*		*%*	*gm*	*gm*
5	15	0	0	8	0	4.7	10.0
5	30	10	0	8	93	1.2	7.1
5	15	0	5	8	0	4.6	9.7
5	15	10	1.5	8	0	4.8	10.0
5	15	10	3	8	0	5.1	10.1
5	30	10	5	8	0	4.6	9.5
10	15	20	0	12	100(2) [1]	—2.8	7.7
10	15	20	5	12	0	2.9	13.7
12	15	20	0	12	100(1)	—2.5	9.2
12	15	20	5	12	0	2.1	14.3
15	16	20	0	13	100	—5.5	8.0
15	15	0	0	13	0	1.9	13.7
17	15	20	0	13	100	—3.8	10.5
17	15	0	0	13	0	1.8	15.9
20	10	20	0	13	100	—4.4	10.1
20	6	20	5	13	0	1.0	14.9

[1] Numerals in parentheses represent the number of animals that died.

The effect of choline. Table 3 shows the effect of choline on the incidence of kidney lesions in rats receiving diet TC-2 plus 2A2MIP. Five-week-old rats receiving diet TC-2 plus 10 mg of the drug per gram of food for 8 days were completely protected against kidney lesions with 1.5, 3 and 5 mg of choline chloride per gram of food. The daily body weight change and food intake were nearly the same as in those animals receiving no 2A2MIP, either with or without added choline chloride.

Of those animals that received diet TC-2 plus the drug without added choline, 93% had hemorrhagic kidneys in the 8-day period. Daily body weight increases and food intakes were on the average 1.2 and 7.1 gm per rat, respectively. Animals receiving choline gained approximately 4.8 gm per rat per day and ate approximately 10 gm of food per rat per day.

Animals 10, 12, 15, 17, and 20 weeks of age were fed diet TC-2 plus 20 mg 2A2MIP for 12 and 13 days. Table 3 shows that all animals in each group had hemorrhagic kidneys at this time. All groups showed body weight losses ranging from 2.5 to 5.5 gm per rat per day. Two animals died in the 10-week-old group and one died in the 12-week-old group.

Choline chloride at a level of 5 mg per gram of diet TC-2 containing 2A2MIP completely protected the 10-, 12-, and 20-week-old animals against kidney lesions. Animals in these age groups receiving choline showed daily body weight gains of from 1.0 to 2.1 gm per rat. Their food intake ranged from 13.7 to 14.9 gm per rat per day. Animals in the 15- and 17-week-old groups consuming diet TC-2 containing neither added 2A2MIP nor added choline chloride gained 1.8 to 1.9 gm per rat per day and ate 13.7 to 15.9 gm of food per rat per day. None of these animals showed hemorrhagic kidneys.

DISCUSSION

Wells ('55a) has shown that renal hemorrhagic degeneration in young male rats on choline-deficient diets was more severe when 2-amino-2-methyl-1-propanol was incorporated into the diet. The condition did not occur when choline was added to the diet. Methionine, betaine and casein were only slightly effective, when they were added in place of choline (Wells, '55b; Mulford, '55). Data presented in this paper show that male rats older than weanling age and consuming a low-choline diet containing 2-amino-2-methyl-1-propanol developed the hemorrhagic condition. Choline was effective in overcoming the drug action in these animals.

Kidney lesions did not occur in the older animals as soon as they did in the weanling age group. The three- and 4-week-

old animals showed the hemorrhagic condition after 4 to 6 days on the diet, while those 6 to 8 weeks old showed the condition only after 8 to 10 days on the diet. Ten milligrams of the drug per gram of food produced severe kidney lesions in all of the three- and 4-week-old animals in 6 or 7 days. This level of drug produced only slightly severe or moderately severe hemorrhages in the kidneys of the 9- to 20-week-old animals in 11 to 13 days. Twenty milligrams of drug per gram of food were required for the severe condition to occur in all animals in these age groups in 11 to 13 days.

Jacobi, Baumann, and Meek ('41) presented evidence that rats were able to synthesize and increase their body stores of choline as they grow. It has been postulated that 2-amino-2-methyl-1-propanol is an inhibitor of choline synthesis in the rat (Wells, '55b; Mulford, '55). The work presented in this paper suggests that when rats are fed complete diets for several weeks, they increase their stores of choline both by dietary intake and synthesis. When they are then placed on low-choline diets containing 2-amino-2-methyl-1-propanol, choline synthesis stops, but renal hemorrhagic degeneration does not develop because of the increased amount of choline stored. When the store of choline is used up, then kidney lesions occur. Investigation in this area is continuing.

SUMMARY

A low-choline diet containing 2-amino-2-methyl-1-propanol was fed to male rats, starting at three to 20 weeks of age. When the drug was fed at a level of 10 mg per gram of food, hemorrhages appeared in the kidneys of the three- to 5-week-old animals in 4 to 7 days, and in the 6- to 8-week-old animals in from 8 to 10 days. Kidney lesions occurred in all animals of the three- to 5-week-old group in from 5 to 7 days and in all animals of the 6- to 8-week-old group in from 6 to 9 days. Death occurred in the three- to 5-week animals in from 6 to 9 days. One animal of the 7-week-old group died on the 12th

day, while none of the 6- and 8-week groups had died by the 10th and 12th day, respectively.

When the diet containing 10 mg of 2-amino-2-methyl-1-propanol per gram of food was fed to 9-, 10-, and 12-week-old rats, only 17, 27, and 13%, respectively, had hemorrhagic kidneys in from 11 to 13 days. None of the rats in this group died during this time. When the diet containing 20 mg of drug per gram of food was fed to animals 9 to 20 weeks of age, 85 to 100% had hemorrhagic kidneys in from 9 to 13 days. Six of the animals in this group died during this period.

During the onset of the hemorrhagic condition there was a decrease in food consumption and growth rate of the animals of all ages. Animals either on low choline diets without the drug or on diets containing both chloine chloride (5 mg per gram of food) and 2-amino-2-methyl-1-propanol (10 and 20 mg per gram of food) ate well, grew well and developed no hemorrhagic kidneys during the experimental period.

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THE INFLUENCE OF ASCORBIC ACID DEFICIENCY IN GUINEA PIGS ON THE SYNTHESIS OF PURINES, SERINE, AND METHIONINE [1]

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The metabolic effects of ascorbic acid deficiency in animals have been summarized in a recent review (Sebrell and Harris, '54). There is considerable evidence that the vitamin is interrelated metabolically with folic acid. For example, anemia frequently accompanies scurvy and is particularly prominent in animals deficient in ascorbic acid and folic acid (May et al., '50; Slungaard and Higgins, '56). Ascorbic acid appears to enhance the conversion of folic acid to citrovorum factor (Nichol and Welch, '50), although recent evidence indicates that this apparent stimulation may be the result of inhibition of the enzymatic destruction of the citrovorum factor (Dinning et al., '56).

In view of these considerations it was desirable to study the influence of ascorbic acid deficiency in animals on the biochemical reactions known to be catalyzed by folic acid derivatives. These include the incorporation of a one-carbon fragment into purines, serine, and the methyl carbon of methionine (Welch and Nichol, '52).

METHODS

Young guinea pigs, weighing initially approximately 100 gm, were given the purified diet described by Reid and Briggs

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('53) with ascorbic acid omitted for one group. The controls were given this same diet supplemented with 2 gm of ascorbic acid per kilogram of diet. After from three to 4 weeks of feeding the animals receiving the deficient diet developed the usual signs of scurvy. Guinea pigs were selected for experiment when they exhibited advanced signs of ascorbic acid deficiency. These signs included weight loss and tenderness of the joints. Upon autopsy all the deficient animals exhibited extensive hemorrhages in the region of the joints.

Tissue nucleic acid concentrations were determined by the procedure of Schneider ('45). For the *in vitro* experiments, tissues were homogenized with two volumes of Robinson's medium (Robinson, '49). One milliliter of homogenate was incubated for 30 minutes at 30°C. with 0.05 ml of carbon^{14} sodium formate solution containing 18,000 counts per minute. The incubation mixture contained the other additions described by Goldthwait and Greenberg ('55), except that the ribose-5-phosphate was omitted since preliminary experiments indicated that it did not influence formate utilization under our conditions. The deproteinization and hydrolysis were carried out as described by Goldthwait and Greenberg (loc. cit.) and 0.1 ml of the supernatant was evaporated on glass planchets for counting. Any unreacted formate would be evaporated by this procedure and the counts obtained from the residue represent formate fixed in such compounds as purines, serine and methionine.

In other experiments, purines, serine, and methionine were isolated separately from the incubation mixture. Purines were isolated as the copper salt after acid hydrolysis (Hitchings and Fiske, '41). Carrier serine and methionine were added to separate aliquots of the supernatant from the purine isolation and then precipitated with alcohol. They were recrystallized to constant radioactivity. All radioactivity measurements were made with an end-window Geiger tube and corrected to infinite thinness.

The influence of ascorbic acid deficiency on the incorporation of formate into purines by the intact animal was also meas-

ured. Normal and deficient guinea pigs were injected with 20 μc of carbon^{14} sodium formate per 100 gm of body weight and were then killed 4 hours later. The combined viscera from each animal was extracted with cold 10% trichloroacetic acid (TCA) to remove the acid-soluble nucleotides and then with 5% TCA at 90°C. to extract nucleic acids (Schneider, '45). The nucleotides were hydrolyzed and the purines isolated as previously described (Dinning et al., '55).

RESULTS AND DISCUSSION

Ascorbic acid deficiency did not alter the ribonucleic acid (RNA) content of liver, spleen, or skeletal muscle nor the deoxyribonucleic acid (DNA) content of liver or small intestine.

TABLE 1

The influence of ascorbic acid deficiency in guinea pigs on tissue nucleic acid concentrations. The results are expressed as milligrams of nucleic acid per gram of tissue with the standard error of the mean. There were three animals per group

	DNA		RNA	
	Control	Deficient	Control	Deficient
Liver	2.52 ± 0.27	1.96 ± 0.13	4.79 ± 0.39	6.67 ± 1.45
Small intestine	3.24 ± 0.17	3.35 ± 0.29	3.64 ± 0.73	4.77 ± 0.29
Skeletal muscle	0.77 ± 0.07	1.07 ± 0.04	2.42 ± 0.34	1.83 ± 0.18

The DNA concentration of skeletal muscle was significantly higher in ascorbic acid-deficient animals than in normal (table 1). In this connection it has been reported that ascorbic acid deficiency leads to pathological changes in skeletal muscle (Sebrell and Harris, '54).

The influence of ascorbic acid deficiency on the utilization of formate by tissue homogenates *in vitro* is illustrated by the data in table 2. The deficiency led to a considerable reduction of formate fixation by liver but was without effect on the activity of the spleen.

After observing the reduction in formate fixation by liver homogenates as a result of ascorbic acid deficiency, experi-

ments were designed to determine which metabolites were affected. The data in table 3 show that ascorbic acid deficiency did not influence the incorporation of formate into purines but led to a considerable reduction in its utilization for the synthesis of serine or methionine. The carbon^{14} activity of purine, serine and methionine accounted for essentially all the formate counts which were fixed.

TABLE 2

The influence of ascorbic acid deficiency on the utilization of C^{14} formate by tissue homogenates. The results are expressed as counts per minute fixed per milligram of tissue with the standard error of the mean. There were 6 animals per group

TISSUE	CONTROL	DEFICIENT
Liver	38.4 ± 5.4	16.8 ± 1.4
Spleen	32.3 ± 1.8	30.1 ± 3.0

TABLE 3

The influence of ascorbic acid deficiency on the incorporation of C^{14} formate into purines, serine, and methionine by guinea pig liver homogenates. The results are expressed as counts per minute fixed per gram of liver. There were 4 animals per group

COMPOUND ISOLATED	CONTROL	DEFICIENT
Purines	98	90
Serine	1888	415
Methionine	245	35

It was somewhat surprising that ascorbic acid deficiency did not affect purine synthesis in view of the anemia which frequently accompanies the condition and also since serine and methionine synthesis were reduced. In order to determine if this lack of effect were due to some deficiency in the *in vitro* system, normal and deficient guinea pigs were injected with carbon^{14} formate and killed 4 hours later. The specific activities of acid-soluble and nucleic acid purines isolated from the viscera of these animals are given in table 4. It may be seen that ascorbic acid deficiency did not influence purine synthesis under these conditions.

The results of these experiments demonstrate that ascorbic acid deficiency in guinea pigs leads to a reduction in the utilization of formate for the synthesis of serine and methionine, reactions which are known to require coenzymes derived from folic acid (Welch and Nichol, '52). However it is also known that folic acid-containing coenzymes are required in the synthesis of purines. If ascorbic acid deficiency results in a depression of the levels of folic acid-containing coenzymes the lack of effect of the deficiency on purine synthesis is difficult to explain. It may indicate that the quantitative coenzyme requirements for these reactions are such that in mild folic acid deficiency the synthesis of serine and methionine is depressed before there is any effect on purine synthesis.

TABLE 4

The influence of ascorbic acid deficiency on the incorporation of injected C^{14} sodium formate into tissue purines of guinea pigs. The results are expressed as counts per minute per micromole of purine. There were three animals per group

	CONTROL	DEFICIENT
Acid soluble purines	1043	1073
Nucleic acid purines	233	209

SUMMARY

Ascorbic acid deficiency in guinea pigs resulted in a reduced utilization of formate in the synthesis of serine and methionine by liver homogenates. The deficiency did not affect the incorporation of formate into purines, either when the formate was incubated with tissue homogenates or injected into the animal.

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STUDIES ON THE BIOSYNTHESIS OF NICOTINIC ACID FROM TRYPTOPHAN IN THE RHESUS MONKEY

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Dietary tryptophan is converted into nicotinic acid in the body of the rat (Rosen, Huff and Perlzweig, '46). While some workers claim that this transformation takes place in the intestine by the activity of the intestinal microorganisms (Ellinger and Abdel Kader, '47; De and Dutta, '51; Ellinger and Benesch, '45) others have shown that it takes place in the tissues (Sydenman et al., '49; Henderson and Hankes, '49; Hundley, '49; Schweigert and Pearson, '47, '48). Several workers have suggested that the liver is the organ concerned in this transformation (Chen et al., '50; Wang et al., '50; Fumio, '51; Priest et al., '51; Ghosh et al., '54). Previously nicotinic acid metabolism in the rhesus monkey was studied by Banerjee and Basak ('55) and it was shown that monkeys when fed extra tryptophan excreted increased amounts of metabolites of nicotinic acid in the urine. The site of the synthesis of nicotinic acid from tryptophan has, however, not been studied in rhesus monkeys. The urinary excretion of some of the metabolites of nicotinic acid, therefore, was estimated in the urine of monkeys fed sulfaguanidine or chloromycetin. Such excretion was also determined in the urine of monkeys whose livers were poisoned by the injection of carbon tetrachloride.

It was shown by Lepkovsky et al. ('43) that pyridoxine was intimately concerned in the metabolism of tryptophan. A

deficiency of pyridoxine in the diets of rats, dogs and mice had been shown to increase the urinary excretion of xanthurenic acid when tryptophan was fed to these animals (Porter et al., '48; Rosen et al., '47) and to decrease the conversion of tryptophan to nicotinic acid as measured by the urinary excretion of N′-methyl nicotinamide (N′MN) (Rosen et al., '47; Schweigert and Pearson, '47; Bell et al., '48; Spector, '48) and blood levels of pyridine nucleotides (Ling et al., '48). Heimberg et al. ('50), however, pointed out that pyridoxine did not appear to be directly involved in the conversion of tryptophan to N′MN by the rat; Sarett ('50) observed that the addition of pyridoxine to a corn diet low in protein, tryptophan and B vitamins had little effect upon the excretion of nicotinic acid, quinolinic acid or N′MN by man and no effect upon the conversion of a small amount of added tryptophan to nicotinic acid. Greenberg and Rinehart ('48) reported the excretion of xanthurenic acid in pyridoxine-deficient monkeys.

Longenecker et al. ('40) observed that rats fed nicotinic acid excreted increased amounts of ascorbic acid in urine. It was also observed by Daft and Schwarz ('52) that symptoms of B vitamin deficiencies in rats could be prevented by feeding ascorbic acid. The effect of feeding pyridoxine hydrochloride and of withdrawal of the ascorbic acid supplement on the urinary excretion of metabolites of nicotinic acid after supplementation with tryptophan was, therefore, studied in rhesus monkeys.

EXPERIMENTAL

The experimental rhesus monkeys were fed the diet with oral supplements of ascorbic acid and vitamins A and D as described by Banerjee and Basak ('55). This diet will be considered as the normal diet. Each animal was placed in a metabolism cage and the urine collected in a bottle containing 2 ml of concentrated hydrochloric acid. Nicotinic acid and amide, nicotinuric acid and N′-methyl nicotinamide (N′MN) were estimated by methods described previously (Chattopadhyay et al., '53). Quinolinic acid was estimated by the method of Sarett ('51) as described by Banerjee and Basak ('55).

The normal urinary excretion of metabolites of nicotinic acid was determined in three monkeys (5, 6 and 7) weighing between 1.6 and 2.5 kg. These animals were fed 1 gm of tryptophan per animal per day mixed with the diet for three consecutive days and the urinary excretion of metabolites of nicotinic acid was estimated on the second, third and 4th days. Four days after the discontinuation of the feeding of tryptophan, when the urinary excretion of these metabolites was normal, each of the animals was again fed 1 gm of tryptophan and 1 gm of sulfaguanidine per day, mixed with the diet, for three days and the urinary excretion of metabolites of nicotinic acid was estimated. Four days after the above experiment each of the animals was again fed daily 10 mg pyridoxine hydrochloride for three consecutive days along with the administration of 1 gm of tryptophan and 1 gm of sulfaguanidine and the metabolites of nicotinic acid were estimated on the second, third and 4th days. After an interval of 4 days, each of the animals received one intraperitoneal injection of carbon tetrachloride in a dose of 0.3 ml per kilogram per day for 6 days and was fed from the 4th day of injection 1 gm of tryptophan per animal per day for three consecutive days and the metabolites of nicotinic acid were estimated on the 5th, 6th and 7th days.

Three rhesus monkeys (8, 9 and 10) weighing between 3.7 and 4.3 kg were taken. After estimating the normal excretion of different metabolites of nicotinic acid the animals were fed 125 mg of chloromycetin palmitate for three consecutive days and those metabolites were again estimated. After an interval of 4 days the animals were fed tryptophan for three days in the usual way and the nicotinic acid metabolites were estimated. Four days after the above experiment each of the monkeys was fed 1 gm of tryptophan and 125 mg of chloromycetin palmitate for 6 consecutive days and the urinary excretion of metabolites of nicotinic acid was estimated on the 5th, 6th and 7th days.

Four rhesus monkeys (11, 12, 13 and 14) weighing between 1.6 and 2.5 kg were taken. After estimating the normal ex-

cretion of metabolites of nicotinic acid following administration of 1 gm of tryptophan per animal per day for three days, ascorbic acid supplements were withdrawn. Ninety days after the withdrawal of the ascorbic acid supplement the monkeys became severely scorbutic. They were then fed 1 gm of tryptophan per animal per day for three days and the metabolites of nicotinic acid were estimated in the urine on the second, third and 4th days.

The animals were all females and DL-tryptophan was the form used.

RESULTS AND DISCUSSION

The results are given in tables 1 and 2.

It will be seen from the tables that the urinary excretion of quinolinic acid and N′MN by monkeys receiving extra tryptophan decreased when the animals were fed sulfaguanidine. This clearly indicates that intestinal microorganisms were concerned with the conversion of tryptophan to nicotinic acid. The urinary excretion of nicotinic acid, quinolinic acid and N′MN by normal and tryptophan-fed monkeys was considerably reduced when chloromycetin was supplied. Administering chloromycetin with tryptophan decreased the excretion of metabolites, but the rate of excretion was still high compared with that observed in controls receiving neither tryptophan nor chloromycetin. These observations suggest that, besides the intestinal tract, tissues of the animal might also be concerned in the biosynthesis of nicotinic acid from tryptophan.

The urinary excretion of the metabolites of nicotinic acid by tryptophan-fed rhesus monkeys was not affected by the intraperitoneal injection of carbon tetrachloride which produced a diminution in the weight of the animals and decreased their food intake. As carbon tetrachloride is likely to damage the liver, the liver of monkeys may not be concerned in the synthesis of nicotinic acid from tryptophan.

Sulfaguanidine destroys intestinal bacteria which might synthesize pyridoxine. The enhanced excretion of the metabolites of nicotinic acid when pyridoxine hydrochloride was

administered to monkeys receiving tryptophan and sulfaguanidine might indicate that pyridoxine was concerned in the conversion of tryptophan to nicotinic acid.

TABLE 1

Twenty-four-hour urinary excretion of metabolites of nicotinic acid by rhesus monkeys fed 1 gm DL-*tryptophan per animal per day for three days with or without any other supplements. (Average of three days' excretion)*

MONKEY NO.	SUPPLEMENT	NICOTINIC ACID AND AMIDE	NICOTINURIC ACID	QUINOLINIC ACID	N'-METHYL NICOTINAMIDE	DIET CONSUMED
		mg	*mg*	*mg*	*mg*	*gm*
5	None	1.10	2.30	1.50	0.18	65
6		0.67	2.10	1.20	0.10	30
7		0.81	2.10	1.56	0.16	65
5	Tryptophan	1.09	2.32	6.23	0.99	62
6		0.74	1.82	2.77	0.32	33
7		0.65	2.28	6.70	0.72	62
5	Tryptophan + sulfaguanidine [1]	1.22	2.31	2.60	0.61	63
6		0.67	1.75	2.14	0.26	29
7		0.82	1.91	4.36	0.59	64
5	Tryptophan + sulfaguanidine [1] + pyridoxine hydrochloride [2]	1.17	3.11	6.17	1.09	69
6		0.62	2.61	3.23	0.73	32
7		1.12	3.48	5.84	1.11	65
5	Tryptophan + CCl_4 injection [3]	0.96	3.05	6.21	0.96	40
6		1.10	2.72	2.71	0.33	19
7		1.14	3.07	6.42	1.08	39
11	Tryptophan	0.46	1.32	5.10	1.28	55
12		0.77	1.85	5.10	1.00	59
13		0.66	2.21	4.31	0.92	55
14		0.60	1.58	5.20	0.95	57
11	Animals made scorbutic by withdrawing ascorbic acid for 90 days and then fed tryptophan	0.82	2.90	4.74	0.72	21
12		0.80	2.41	5.04	0.78	20
13		0.94	3.30	6.56	1.10	18
14		0.88	3.20	6.48	0.92	18

[1] One gram sulfaguanidine fed per animal per day for three days.

[2] Ten milligrams pyridoxine HCl fed per animal per day for three days.

[3] Three-tenths milliliter carbon tetrachloride injected intraperitoneally per animal per day for 6 days.

The urinary excretion of metabolites of nicotinic acid after feeding tryptophan did not vary consistently in all the monkeys when they developed scurvy. This indicated that ascorbic acid nutrition is not primarly concerned in the conversion of tryptophan to nicotinic acid.

TABLE 2

Twenty-four-hour urinary excretion of metabolites of nicotinic acid by rhesus monkeys fed chloromycetin palmitate. (Average of three days' excretion)

MONKEY NO.	SUPPLEMENT	NICOTINIC ACID AND AMIDE	QUINOLINIC ACID	N'-METHYL NICOTINAMIDE	DIET CONSUMED
		mg	*mg*	*mg*	*gm*
8	None	0.69	4.81	0.10	93
9		0.73	4.75	0.10	91
10		0.67	4.90	0.08	96
8	Chloromycetin (125 mg per animal per day for three days)	0.42	3.81	0.08	89
9		0.36	3.29	0.06	82
10		0.36	3.29	0.05	89
8	Tryptophan (1 gm per animal per day for three days)	0.65	11.39	0.46	94
9		0.45	7.14	0.44	79
10		0.74	11.34	0.55	92
8	Tryptophan + chloromycetin (1 gm and 125 mg respectively fed per animal per day for 6 days)	0.56	9.17	0.31	89
9		0.46	5.31	0.25	84
10		0.67	6.45	0.32	90

SUMMARY

Nicontinic acid and amide, quinolinic acid and N'-methyl nicotinamide (N'MN) were estimated in the urine of normally-fed and tryptophan-fed rhesus monkeys after they had received chloromycetin palmitate. Chloromycetin reduced the urinary excretion of nicotinic acid and amide, quinolinic acid and N'MN in both normal and tryptophan-fed monkeys.

Nicotinic acid and amide, nicotinuric acid, quinolinic acid and N'MN were estimated in the urine of rhesus monkeys after

the administration of pyridoxine hydrochloride in tryptophan- and sulfaguanidine-fed monkeys. The pyridoxine supplement led to an increased excretion of nicotinuric acid, quinolinic acid and N'MN by these animals. It has been suggested that pyridoxine is needed for the conversion of tryptophan to nicotinic acid.

The urinary excretion of metabolites of nicotinic acid was also estimated after the administration of tryptophan in monkeys which were injected intraperitoneally with carbon tetrachloride to damage the liver. The excretion of metabolites of nicotinic acid in the urine of monkeys poisoned with carbon tetrachloride did not vary consistently. Liver, therefore, was not the site of the synthesis of nicotinic acid from tryptophan.

The urinary excretion of metabolites of nicotinic acid was also estimated in normal and scorbutic monkeys after they were fed tryptophan. Ascorbic acid deficiency had no effect on the excretion of these metabolites.

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THE DIGESTION AND ABSORPTION OF FAT IN DOG AND MAN [1,2]

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Despite a century of investigation, the mechanisms of fat digestion and absorption are not fully understood. The earliest workers, who possessed few analytical tools for the characterization of lipid material, confined their work mainly to experiments *in vivo*. Later workers had many more analytical procedures at their disposal, but directed much of their attention to the action of lipases *in vitro*. Detailed observations on the course of lipolysis *in vivo* are, therefore, rather scanty. Furthermore, conclusions regarding intestinal lipolysis *in vivo* were usually based on data obtained from the analysis of pooled samples from the entire length of the small intestine. Although the low gastric lipase activity reported by most workers minimizes the quantitative importance of this enzyme, the importance of gastric lipase is still not fully determined. It is necessary, therefore, to determine the extent to which fat derived from a particular test meal is digested in the stomach in order that intestinal lipolysis may be more fully evaluated for the same test meal. The purpose of the present work was to investigate in more detail the composition of fat

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[2] The data in this paper were taken from a thesis submitted by Leon K. Knoebel to the Graduate School of the University of Rochester in partial fulfillment of the requirements for the degree Doctor of Philosophy, June 1955.

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in the stomach and in different regions of the small intestine of the dog and man after feeding various fat test meals. It was also hoped that a better understanding of the absorptive processes taking place would be gained by making comparisons between these studies *in vivo* and certain investigations performed by other workers *in vitro*.

METHODS

Dogs were fasted at least 18 hours before being fed a test meal and allowed to remain in their cages thereafter for three hours with free access to water. They were then killed, the abdomen was opened and after placing clamps at the pylorus and cardia, the small intestine was divided somewhat arbitrarily into duodenum, jejunum, and ileum. The duodenum was considered to extend from the pylorus to the ligament of Treitz; the remainder of the intestine from this point to the cecum was divided into halves, the proximal portion being called jejunum and the remainder ileum. After first gently milking out most of their contents, the stomach and each segment of intestine were slit longitudinally and thoroughly washed with distilled water.

In two dogs under anesthesia [4] the jejunum was transected approximately 50 cm below the ligament of Treitz and the proximal end was joined to the distal segment approximately 20 cm below the point of transection by an end-to-side anastomosis. The distal end was passed through a stab wound in the abdominal wall and sutured firmly in place. For collection of intestinal contents a double-lumen balloon catheter was inserted in the fistula. The balloon was placed just below the anastomosis and inflated to prevent the onward passage of the contents which were continuously removed by aspiration at —10 cm of water pressure into a collecting cylinder immersed in an ice bath. The dogs were fasted at least 18 hours before feeding the test meal and the intestinal contents were collected until the upper gastrointestinal tract

[4] Nembutal.

was empty again. Every 30-minute sample was mixed with the solvent mixture described below.

Observations were made also on the gastric and intestinal hydrolysis of fat in human subjects. Subjects in the post-absorptive state swallowed a Levine tube, ingested the test meal and at the end of the experimental period (one-half to one hour) the gastric contents were withdrawn as completely as possible. In other subjects a Rehfuss tube was passed into the small intestine after which the test meal was ingested. The position of the tube as established by fluoroscopy indicates that the samples collected by continuous aspiration for one and three-fourths to two and three-fourths hours were obtained from the duodenum.

The procedure used for the isolation of lipid from the gastrointestinal contents was similar to that used by Desnuelle and Constantin ('52). Gastric and intestinal contents were mixed separately in 4 times their own volume of a mixture consisting of equal volumes of ethyl ether and 95% ethanol to precipitate protein, inactivate lipases and extract the lipids. This preparation was filtered and the residue re-extracted a number of times with the solvent mixture. The combined extracts were then concentrated under diminished pressure at temperatures below 50°C. The concentrate was transferred to a separatory funnel and extracted three times with ethyl ether. The combined ether extracts were washed with water, dried over anhydrous sodium sulphate and filtered. The filtrate containing the purified lipids was evaporated on a steam bath under nitrogen to constant weight.

The extent of fat hydrolysis in the stomach and intestine was ascertained by the quantitative determinations of free fatty acids and monoglycerides. Free fatty acids were titrated in 95% ethanol with alcoholic 0.1N KOH to the phenolphthalein endpoint. Monoglycerides were determined by the iodometric technique of Pohle and Mehlenbacher ('50). Since this method is specific for adjacent hydroxyl groups, only the 1-monoglycerides are included and any 2-monoglycerides present are excluded. The 2-monoglycerides, however, could never

be detected in the lipid by specific tests. These results are in contrast to those of Mattson et al. ('52), who found that 2-monoglycerides do appear during intestinal digestion. However, special precautions were taken by these workers to prevent the isomerization of the 2-form to the 1-form during the lipid isolation procedure. Herting et al. ('55) were able to detect only 1-monoglycerides in the intestinal lipid of rats and suggested that conversion of the 2-isomer to the 1-isomer may have occurred during removal of the solvent from their samples. Since this conversion takes place quite readily under a variety of conditions, it is likely that moderate heating as used in our lipid isolation procedure was sufficient to cause complete isomerization of the 2-form to the 1-form. Consequently, if both forms of monoglycerides were present in the gastrointestinal lipid they are probably both included in the final values presented in this work. That part of the recovered lipid not accounted for as free fatty acids and monoglycerides was assumed to consist of diglycerides and triglycerides.

If dogs were forced to eat test meals which they disliked, the food tended to remain in the stomach for long periods of time. In an attempt to produce meals of acceptable consistency and palatability, sucrose, casein, and cellulose flour were added to the test fat in varying proportions. In the acute experiments 9 dogs were fed a test meal consisting of 40 gm of sucrose and 20 gm of casein mixed with a little water, 5 dogs received 40 gm of cottonseed oil triglycerides and 100 gm of sucrose, and 9 dogs were fed a test meal containing 20 gm of monoglycerides, 15 gm of sucrose, and 15 gm of cellulose flour. The monoglycerides [5] consisted essentially of the 1-monoester of linoleic acid. The fistula dogs were fed test meals of the following composition: 25 gm sucrose and 25 gm of casein; 25 gm cottonseed oil, 25 gm sucrose, and 25 gm casein; 25 gm monoglycerides, 15 gm casein, 25 gm sucrose, and 15 gm cellulose flour; 25 gm diglycerides and 40 gm sucrose. The diglycerides [5] were commercially prepared from

[5] The monoglycerides and diglycerides were provided by the Distillation Products Industries, Incorporated, Rochester, New York.

cottonseed oil and contained the same fatty acids in the same proportions as found in cottonseed oil. Gastric contents were recovered from 6 human subjects fed 150 gm of vanilla ice cream containing 15 gm of butterfat triglycerides. Duodenal contents were collected from 6 other human subjects fed the ice cream test meal.

RESULTS AND DISCUSSION

Acute experiments. The composition and weights of fat recovered from the stomach and various portions of the small intestine after feeding fat-free, cottonseed oil, and monoglyceride test meals to dogs, which were killed three hours later, are given in table 1. Non-fat test meals were fed in order to obtain an estimate of the quantity of endogenous

TABLE 1

Weights and composition[1] *of lipid recovered from gastrointestinal contents of the dog*

TEST MEAL	SAMPLE FROM		TOTAL LIPID WEIGHT	MONOGLYCERIDES	FREE FATTY ACIDS	DI- AND TRIGLYCERIDES[2]
			gm	%	%	%
Non-fat	Test meal		0.0	0.0	0.0	
	Stomach	(6)[3]	0.095 ± 0.061[4]	6.6 ± 1.7	20.0 ± 1.9	
	Duodenum	(9)	0.029 ± 0.009	....	34.5 ± 2.7	
	Jejunum	(9)	0.078 ± 0.027	13.9 ± 1.6	42.9 ± 6.7	
	Ileum	(9)	0.053 ± 0.015	15.6 ± 1.6	44.4 ± 6.9	
Cotton-seed oil	Test meal		40.0	0.4	0.1	99.5
	Stomach	(5)	26.1	1.1 ± 0.3	3.1 ± 0.9	95.8 ± 0.5
	Duodenum	(5)	0.309 ± 0.167	4.9 ± 0.4	17.3 ± 2.2	78.2 ± 2.4
	Jejunum	(5)	0.606 ± 0.299	9.0 ± 1.5	33.1 ± 7.4	57.8 ± 7.4
	Ileum	(5)	0.185 ± 0.083	12.8 ± 2.9	47.3 ± 7.2	39.9 ± 14.1
Monoglyc-eride	Test meal		20.0	92.5	0.9	6.6
	Stomach	(9)	13.1	86.3 ± 0.6	2.8 ± 0.3	10.9 ± 0.5
	Duodenum	(9)	0.178 ± 0.040	27.7 ± 3.0	43.1 ± 2.3	29.2 ± 2.6
	Jejunum	(9)	0.390 ± 0.120	19.3 ± 1.9	43.0 ± 3.0	37.6 ± 3.5
	Ileum	(7)	0.174 ± 0.034	21.4 ± 2.6	46.3 ± 5.1	32.3 ± 7.6

[1] All values represent percentage composition by weight.

[2] Determined by difference.

[3] Number of samples given in parentheses.

[4] Mean ± standard error of the mean.

lipid contributed by the digestive tract. Very small amounts of endogenous lipid were recoverd from the stomach of 6 dogs fed the fat-free meal with the result that lipid sufficient for monoglyceride analyses could be obtained from only three of the dogs. Appreciable quantities of endogenous lipid were present in all regions of the intestine examined, the composition of which was not significantly different in the various segments. The part of the lipid not accounted for as monoglycerides and free fatty acids probably consisted of higher glycerides and unsaponifiable matter.

Endogenous lipid, while constituting a negligible part of the gastric lipid, probably did make up an appreciable part of the total lipid recovered from the intestine after feeding either fat meal. It was necessary, therefore, to calculate the extent to which the presence of endogenous lipid might alter the composition of intestinal lipid presumably derived from dietary sources. Corrections made for the contribution of endogenous lipid to the various components making up the total lipid recovered from the intestine, after feeding either of the fat meals, scarcely altered the composition of the intestinal lipid and hence the uncorrected values are given in table 1.

As judged by the presence of only small amounts of free fatty acids in the gastric lipid, cottonseed oil and monoglycerides were only slightly hydrolyzed in the stomach. Thus, interpretation of results obtained on intestinal lipolysis is not complicated by an extensive gastric lipolysis. Since only small amounts of hydrolytic products were present in the gastric lipid of the dogs, it was necessary to ascertain whether these products appeared as the result of enzyme activity or were formed during the isolation of the lipid from gastric contents. Every test meal used in these studies was mixed with 100 ml of distilled water, brought to approximately pH 3 by addition of HCl, and incubated at 37°C. for three hours. The composition of the test meal lipids was unchanged by this treatment; free fatty acids and monoglycerides were not formed during the isolation procedure and it seems fair to

assume, therefore, that their presence in the gastric lipid was due to the action of lipase.

As cottonseed oil triglycerides passed through the small gut, lipolysis progressed steadily as manifested by an increased percentage of monoglycerides and free fatty acids. On the basis of computation by difference a corresponding decrease occurred in the percentage of diglycerides and triglycerides. Some useful comparisons can be made between

TABLE 2

Pancreatic lipase hydrolysis[1] *of triolein and diolein in vitro*

SUBSTRATE	HOURS	MONO-GLYCERIDES	FREE FATTY ACIDS	DI- AND TRI-GLYCERIDES	COMPLETE HYDROLYSIS[2]
		%	%	%	%
Triolein[3]	0	0	0	100	0
	¼	7	21	73	2
	½	14	36	53	3
	1	17	51	34	10
	1½	17	64	20	29
Triolein[4]	0	0	0	100	0
	½	5	24	71	12
	1	4	40	56	28
	2	4	48	48	37
Diolein[4]	0	0	0	100	
	½	14	22	64	
	1	18	29	53	
	2	10	46	45	

[1] All values represent percentage composition by weight.
[2] Calculated from the amount of glycerol liberated.
[3] Calculated from the data of Desnuelle ('51).
[4] Calculated from the data of Borgstrom ('54).

the composition of lipid recovered in these experiments *in ivo* and that obtained by the action of pancreatic lipase *in itro* as reported by Desnuelle ('51) and Borgstrom ('54). These workers studied the hydrolysis of triolein, a triglyceride chemically and physically similar to cottonseed oil. The composition of lipid obtained by them during the first one and one-half to two hours of hydrolysis *in vitro* is recalculated in table 2. Normally, not much longer than two hours are re-

quired for intestinal contents to pass from the pylorus to the ileocecal value. If the results obtained by analyses of the contents of the duodenum, jejunum and ileum be considered as representing different time intervals, the results obtained *in vivo* (table 1), excepting the monoglycerides, agree fairly well with those obtained *in vitro* (table 2). The proportions of free fatty acids and combined diglycerides and triglycerides are quite similar in the two types of experiments. Approximately the same length of time was required to produce lipid of very similar composition in both instances, which suggests that the nature of fat hydrolysis may be the same in both.

Appreciable amounts of partial glycerides, as well as free fatty acids, were formed in one to two hours of hydrolysis *in vitro* but only approximately one-third of the triglycerides were completely hydrolyzed during this time (table 2). If lipolysis proceeds by the same mechanism and at approximately the same rate *in vivo* and *in vitro,* the amount of fat completely hydrolyzed in the small intestine is also limited. Such results would lend support to the idea of Frazer ('46) that fat is only partially hydrolyzed in the intestine and absorbed in part as minute particles consisting of free fatty acids, triglycerides and partial glycerides. It might be argued further that in order to maintain the same composition of lipid in the intestine as in the test tube all lipid components are absorbed in the proportions in which they exist as the result of digestion at any point in the intestine. In this way the composition of the lipid in the intestine, where both digestion and absorption occur simultaneously, would be similar to that in the test tube, where only digestion can take place.

If the comparisons between the hydrolyses *in vivo* and *in vitro* are valid, the present data do not conform to the concept of fat digestion which necessitates a complete breakdown of triglycerides to free fatty acids and glycerol and their absorption as such (Verzar and McDougall, '36). It is not impossible, however, that a specific hydrolytic product is absorbed in the intestine with the result that the lipids

produced *in vivo* and *in vitro* are similar. If this is the case, then the resulting similarity in composition is probably coincidental and the mechanisms by which fat is digested *in vivo* and *in vitro* are not comparable.

When monoglycerides were fed, there was a large decrease in the amount of monoglycerides and a correspondingly large increase in the amount of free fatty acids present in the intestinal lipid as fat passed from the stomach into the duodenum. It was also found that the combined diglycerides and triglycerides were increased and, in the ileum were 5 times as concentrated as in the test meal (table 1). It appears that synthesis and hydrolysis proceeded simultaneously, i.e., diglycerides and triglycerides were formed at the same time that monoglycerides were being hydrolyzed.

In order to gain a better picture of the molecular transformations taking place, the composition of the gastrointestinal lipid was recalculated in terms of millimoles of component per gram of intestinal lipid and plotted against the percentage of intestinal length. The millimolar proportions of diglycerides and triglycerides were unknown, but if this combined fraction were assumed to consist entirely of either diglycerides or triglycerides, millimolar values could be calculated for each component. Values falling between the extremes represent mixtures of these two glycerides. If the composition of the intestinal lipid reflects the manner in which lipase acts on the monoglycerides, certain conclusions are justified concerning the digestion of monoglycerides *in vivo*. The molecular relationships are presented in figure 1.

As judged by the large number of free fatty acid molecules liberated in the duodenum, most of the decrease of monoglycerides could be accounted for by the hydrolysis of monoglyceride to free fatty acid and glycerol. Hydrolysis predominated over resynthesis in this segment. In the transit of fat through the remainder of the intestine there was little change in the molecular relationships of the components and it appeared that an equilibrium had been reached among the hydrolytic and synthetic reactions of triglycerides, diglyc-

erides, and monoglycerides. Perhaps after the initial rapid hydrolysis, a system was formed which was not suitable for optimal digestion by the lipase. If this were the case, then it is possible that an accumulation of hydrolytic products may slow down the digestion process in the intestine sufficiently so that hydrolysis does not proceed at a rate rapid enough to permit complete hydrolysis of fat and its absorption as free fatty acid only.

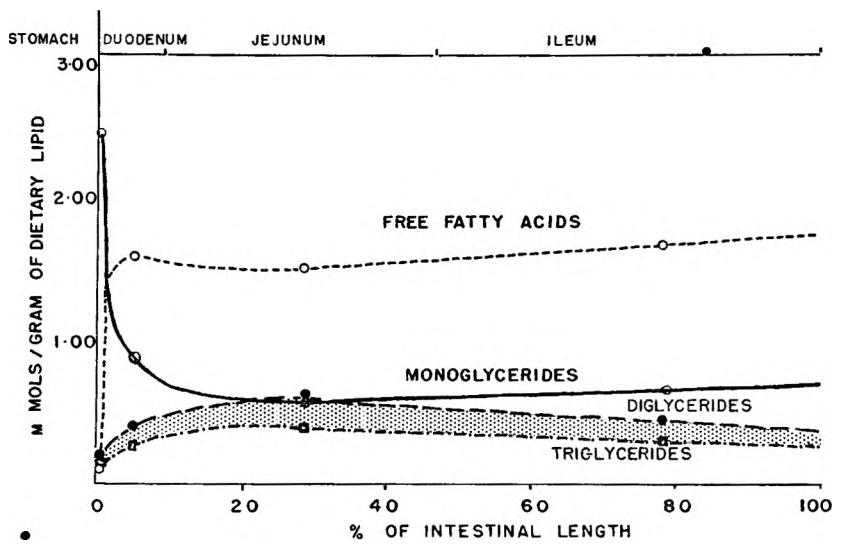

Fig. 1 Composition of gastrointestinal lipid after feeding monoglyceride test meal. In calculating the molecular relationships of the lipid components, all glycerides and free fatty acids were assumed to be made up entirely of oleic acid. The lipid not accounted for as free fatty acids and monoglycerides was assumed to consist entirely of either triglycerides or diglycerides. The values falling between these extremes (stippled area) represent various possible mixtures of these two glycerides.

Some comparisons can be made between the composition of intestinal lipid recovered after feeding the two structurally different glycerides. The composition of the intestinal lipid recovered after feeding each type of test meal, although quite different in the duodenum, gradually became more alike as fat passed through the small gut (table 1). When the lipid

reached the ileum, the lipid components were present in very similar proportions regardless of the glyceride fed. This same phenomenon occurred with respect to the monoglycerides and free fatty acid content of endogenous lipid. These data may mean that a certain lipid system tends to be formed by the action of lipolytic enzymes and the ease with which such a system is formed probably depends on the type of fat fed.

TABLE 3

The digestion[1] *and absorption of various lipids, fed and recovered from jejunal fistula dogs*

TEST MEAL[2]	DOG NO.	NO. OF SAMPLES	TOTAL LIPID RECOVERED	ABSORPTION	MONOGLYCERIDES	FREE FATTY ACIDS	DI- AND TRIGLYCERIDES[3]
			gm	%	%	%	%
Non-fat	1	2	0.255 ± 0.047		16.2 ± 3.5[4]	60.0 ± 7.2	
	2	5	0.213 ± 0.024		10.3 ± 0.7	51.4 ± 3.6	
Cotton-seed oil	1	13	7.5 ± 1.1	69.9 ± 4.3	6.5 ± 0.4	29.3 ± 2.2	64.4 ± 2.4
	2	13	11.2 ± 1.7	55.0 ± 7.0	10.6 ± 0.8	25.7 ± 3.4	63.7 ± 3.9
Diglyceride	1	6	9.5 ± 1.5	61.7 ± 6.0	15.0 ± 1.0	22.3 ± 1.9	62.8 ± 2.4
	2	6	11.6 ± 1.5	29.9 ± 6.2	11.6 ± 1.5	12.3 ± 1.2	76.2 ± 2.5
Monoglyceride	2	5	3.1 ± 0.5	85.3 ± 2.9	30.1 ± 4.3	54.6 ± 4.8	15.4 ± 1.1

[1] All values represent percentage composition by weight.
[2] All lipid test meals contained 25 gm of lipid.
[3] Determined by difference.
[4] Mean ± standard error of the mean.

Fistula experiments. The digestion and absorption of fats were studied in two dogs possessing Maydl jejunostomies. The lipid recovered from the fistula of these animals represents unabsorbed and partially digested material remaining after passage of the test meal through the small gut to the proximal jejunum where the fistula was located. The results of these experiments are given in table 3.

Only small quantities of endogenous lipid were recovered when fat-free test meals were fed and it was considered negligible when compared with the total amount of lipid recovered when fats were present in the test meal. The per-

centages of monoglycerides and free fatty acids contained in the endogenous lipid remained fairly constant.

The composition of fat recovered from the intestine of the fistula dogs after feeding cottonseed oil was similar to that recovered from the jejunum of the dogs fed the same fat in the acute experiments. Consideration of individual collections showed that the relationship between individual components was the same as in the acute experiments; as the percentage of diglycerides and triglycerides decreased, the percentages of monoglycerides and free fatty acids increased. Dogs 1 and 2 absorbed 69.9 and 55.0% respectively of the ingested cottonseed oil, indicating that the duodenum and proximal jejunum must absorb fat quite efficiently.

It was interesting to note that, while considerable amounts of fat were absorbed in this short length of intestine, much of the recovered fat consisted of diglycerides and triglycerides. Probably only a short time is required for the test meal to travel from the stomach to the fistula. This is substantiated by the fact that intestinal contents usually began to appear in 5 to 10 minutes after feeding the test meal. This is a short time for lipase action and it is doubtful that hydrolysis could have proceeded to the extent that all the fat absorbed consisted entirely of free fatty acids. The composition of the lipid recovered from the fistula dogs closely resembled that produced during the first one-fourth to one-half hour of the hydrolyses of triglycerides *in vitro* as reported by Desnuelle ('51) and Borgstrom ('54) (table 2). The fact that approximately the same length of time was required to produce lipid of very similar composition in both types of studies suggests that fat is hydrolyzed in much the same manner in both instances. The experiments *in vitro* show that not enough free fatty acids are liberated in such a short time to account for the large amounts of fat being absorbed.

In order to investigate further whether lipolysis *in vitro* proceeds in the same manner as *in vivo*, a diglyceride test meal was fed to the fistula dogs. The composition of the lipid recovered after feeding diglycerides was found to resemble

closely the composition of lipid produced during the first one-half hour of the hydrolysis of diolein *in vitro* (table 2). The studies made with diglycerides once again indicate that fat was hydrolyzed in approximately the same manner *in vivo* as *in vitro*.

In general, the composition of the lipid recovered from the jejunal fistula dog fed monoglycerides was similar to that recovered from the intestine of dogs fed monoglycerides in acute experiments. A large quantity of monoglycerides was hydrolyzed and much free fatty acid was liberated during passage through the duodenum and proximal jejunum. Smaller percentages of diglycerides and triglycerides were present

TABLE 4

Gastrointestinal lipolysis [1] *in man*

SAMPLE FROM	HOURS	RECOVERED LIPID	MONO-GLYCERIDES	FREE FATTY ACIDS	DI AND TRI-GLYCERIDES [2]
		gm	%	%	%
Ice cream (test meal)		15	1.0	0.5	98.5
Stomach (6) [3]	½–1	8.5	2.0 ± 0.2 [4]	7.0 ± 0.8	91.0 ± 2.2
Duodenum (6)	1¾–2¾	2.9	4.9 ± 0.8	26.0 ± 3.5	69.1 ± 4.4

[1] All values represent percentage composition by weight.

[2] Determined by difference.

[3] Number of samples given in parentheses.

[4] Mean ± standard error of the mean.

than were found in the intestinal lipid of the dogs in the acute experiments, but it appeared likely that a synthesis of these products took place through esterification of partial glycerides with free fatty acid.

Human experiments. Gastric and duodenal contents were recovered from human subjects after feeding 150 gm of vanilla ice cream containing 15 gm of butterfat triglycerides. The results of these experiments appear in table 4. Somewhat more free fatty acid (7.0%) appeared in the gastric lipid when butterfat triglycerides were fed to human subjects than when cottonseed oil was fed to dogs (3.1%). The butterfat was present in emulsified form and consisted of triglycerides containing small amounts of short-chain fatty acids. Both

of these factors have been reported to enhance gastric lipolysis (Schonheyder and Volquartz, '45).

The composition of lipid recovered from human duodenal contents after feeding the ice cream test meal (table 4) was similar to that found in the small gut of the dog after feeding cottonseed oil triglycerides. The relationship between individual components was the same as that found when dogs were fed triglyceride fat; as the percentage of monoglycerides and free fatty acids increased, the percentage of diglycerides and triglycerides decreased. The monoglyceride values were considerably lower than those reported by Kuhrt et al. ('52); they reported 38% and 50% of the lipid as monoglyceride in the contents recovered from the duodenum of two human subjects but it is possible that hydrolysis continued for some time after collection of their samples.

SUMMARY

Small amounts of endogenous lipid of fairly constant composition were recovered from all parts of the small intestine of dogs fed fat-free test meals.

When cottonseed oil was fed, the composition of lipid recovered from the small intestine of dogs killed after three hours closely resembled that reported by others during the first one to two hours of the hydrolysis of triolein *in vitro*. The composition of lipid recovered from jejunal fistula dogs fed cottonseed oil was similar to that reported by others as being produced during the first one-fourth to one-half hour of the hydrolysis of triolein *in vitro*.

Fat recovered from fistula dogs fed diglycerides was very similar in composition to that produced during the first one-half hour of the hydrolysis of diolein *in vitro*. Fat may be hydrolyzed, therefore, in much the same manner *in vivo* as *in vitro*.

Hydrolysis of monoglycerides fed to dogs was very rapid in the duodenum. The proportion of diglycerides and triglycerides was greatly increased in the intestine, suggesting that synthesis and hydrolysis occur simultaneously.

Gastric lipolysis of cottonseed oil and monoglycerides in the dog was slight, while butterfat was somewhat more digested in the stomach of humans.

The composition of lipid recovered from the small intestine of man fed butterfat triglycerides was very similar to that found in the small gut of dogs fed cottonseed oil.

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A COMPARATIVE STUDY OF THIAMINE-SPARING AGENTS IN THE RAT

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Since it was first shown by Evans and Lepkovsky ('28) that fat in the diet lowered the dietary requirement for thiamine, it has been found that protein (Banerji, 41), antibiotics (Lih and Baumann, '51) and large amounts of ascorbic acid (Daft and Schwartz, '52), have a similar effect. It has been commonly assumed that fat lowers the requirement for thiamine because fat is metabolized by a pathway that does not involve limiting amounts of thiamine pyrophosphate. It has also been assumed that antibiotics spare thiamine by alteration of intestinal flora. However, the mode of action of none of these sparing agents is known with certainty.

The present studies were designed to compare these sparing agents with respect to: (1) their effect on the growth curves of rats; (2) whether or not they are effective in combination; and (3) their effect on the thiamine content of the body.

METHODS

Weanling male rats of the Sprague-Dawley strain, weighing between 25 and 65 gm were used throughout these experiments. The composition of the diets used is shown in table 1. The diets were approximately equivalent with respect to calories, vitamins and minerals. Where penicillin, streptomycin and aureomycin were added to the diets, the amount used was

50 mg/kg. Ascorbic acid, where used, was added to the formulations shown in table 1 in the amount of 138 (high-ascorbic acid diet) or 69 gm (low-ascorbic acid diet) replacing an equal weight of sucrose, fat or protein in the standard, high-fat, and high-protein diets respectively. Where thiamine was given, it was administered intraperitoneally in all cases. The casein used contained 0.15 ± 0.05 μg/gm of thiamine as determined microbiologically (Sarett and Cheldelin, '50), 0.2 μg/gm as determined by the thiochrome method. Pyrithiamine, where used, was injected in the amount of 50 μg per day.

TABLE 1

Composition of diets

CONSTITUENT	AMOUNT OF PARTS BY WEIGHT		
	Standard	High fat	High protein
Casein [1]	600	600	2300
Sucrose	1700	....	....
Hydrogenated vegetable oil [2]	275	1067	275
Salts [3]	100	100	100
Thiamine content,[4] μg/gm	0.03	0.05	0.13
Vitamins [5]			

[1] Nutritional Biochemicals Co., "Vitamin-Low."

[2] "MFB," Wesson Oil and Snowdrift Sales Co.

[3] Jones and Foster ('42).

[4] Calculated from thiamine content of casein.

[5] The following vitamins were added: riboflavin, 75 mg; pyridoxine hydrochloride, 75 mg; calcium pantothenate, 100 mg; choline chloride, 2 gm; vitamin A, 130,000 I.U.; vitamin D, 26,000 I.U.; α-tocopherol, 0.5 mg; inositol, 2 gm; niacin, 100 mg; biotin, 7.5 mg; folic acid, 7.5 mg; *p*-aminobenzoic acid, 1 gm; 2-methylnaphthoquinone, 150 mg; and vitamin B_{12}, 30 μg.

For reasons discussed below, the measure of weight change chosen was weight at the end of 4 weeks on the diets minus that at the end of the first week. The thiamine intake was calculated from food consumed plus any injected thiamine.

After 4 weeks on the experimental diets, the amount of thiamine in the liver and in the rest of the carcass, excluding the skin, was determined microbiologically by the method of Sarett and Cheldelin ('50).

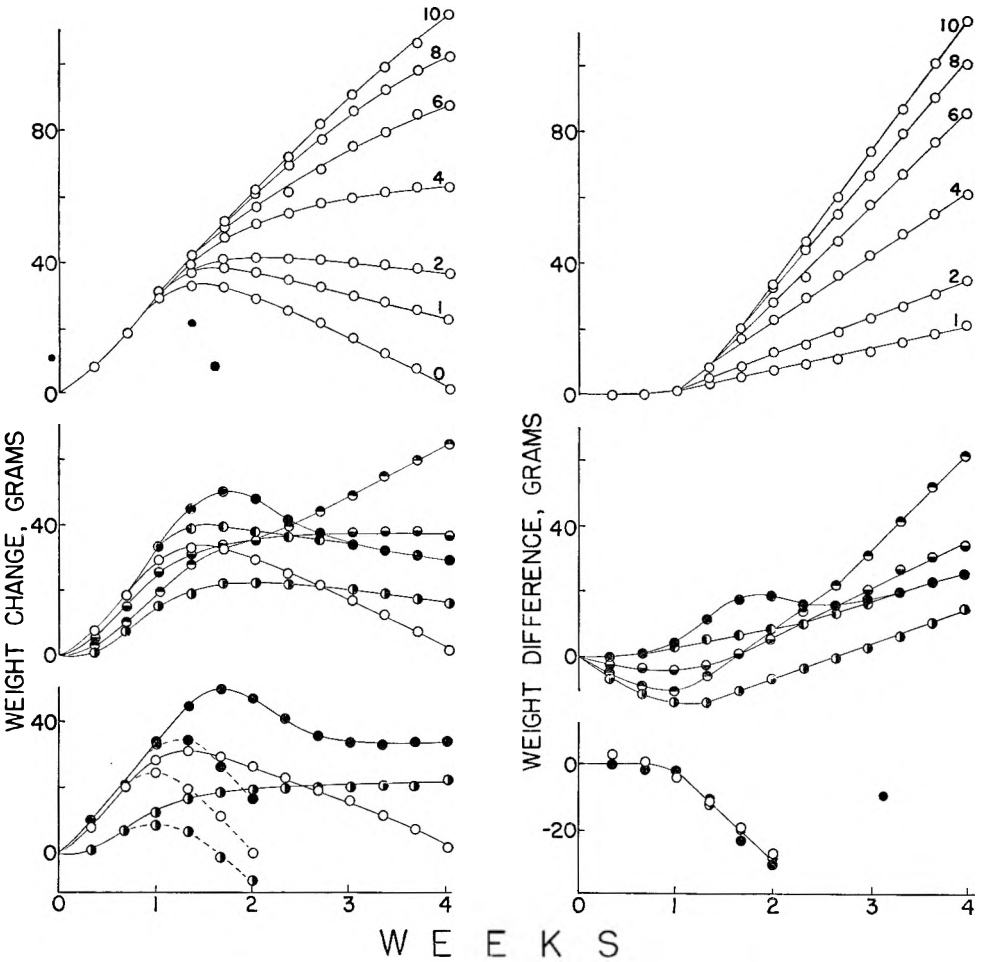

Fig 1 Effects of thiamine, sparing agents, and pyrithiamine on growth of rats. Diets shown are: ○, standard; ●, fat; ◑, protein; ◐, standard plus penicillin; ◒, standard plus low-ascorbic acid; ◓, standard plus high-ascorbic acid. Upper left: Effect of graded levels of thiamine on weight change. Numbers represent micrograms of thiamine injected per day. Upper right: Difference curves obtained by subtracting weight change with no thiamine from that on graded levels of thiamine. Center left: Effect of sparing agents on weight change. Center right: Difference curves of sparing agents. Lower left: Effect of pyrithiamine (dotted lines) on weight change. Lower right: Difference curves obtained by subtracting weight change with pyrithiamine from weight change without pyrithiamine.

RESULTS

First of all, a suitable measure of weight change was desired for these experiments. In figure 1 are shown the growth of rats on the standard diet receiving graded amounts of thiamine, and that of those receiving various thiamine-sparing agents. In no case were the curves linear, and in the case of the high-protein and ascorbic acid diets, there was an initial growth retardation which was apparently due to distaste for these diets. This latter effect precluded the use of initial weights in assessing growth.

One effective method of comparing growth curves is to subtract the weight of animals on a basal diet from the weight of experimental animals. In figure 1 are shown difference curves of growth in these rats, calculated by subtracting the mean weight of rats on the standard diet from the other mean weights. It will be noted that graded amounts of thiamine produced linear difference curves between the first and 4th weeks. Consequently weight change in this period was considered to be a suitable measure of the effects of thiamine.

Weight change between the first and 4th week appeared also to describe fairly well the effects of sparing agents, since initial growth retardation had largely disappeared by the end of the first week. Protein gave an essentially linear difference curve. Ascorbic acid and penicillin did also, except that after the middle of the third week, many animals appeared to show slightly improved growth. The sinuosity in the curve produced by fat was definite and reproducible, and was caused by the tendency of animals on a high-fat diet to retain a normal rate of gain for a longer period of time than animals on the standard diet, followed by an initially rapid decline in weight. However, since this deviation from linearity occurred almost wholly within the period of the first to 4th week, the proposed measure appeared to express fairly the growth performance.

The relation of thiamine intake to weight change between the first and 4th week on the standard diet is shown in figure 2. The relation is linear between 2 and 6 μg of thiamine intake per day. By comparison with this curve, the "thiamine intake for equivalent growth" was calculated in the case of the sparing agents. The "thiamine-sparing" of the agents could

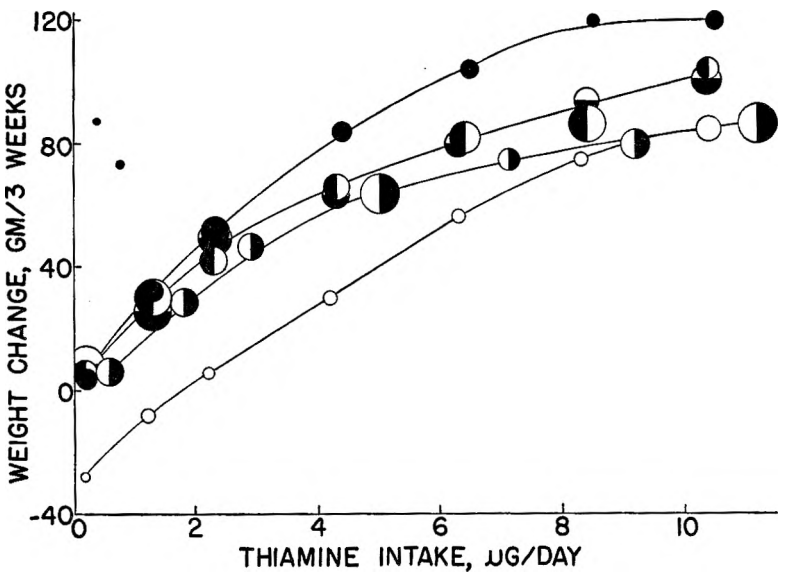

Fig. 2 Effects of injected thiamine on weight change of rats fed the standard diet or thiamine-sparing diets. Symbols are the same as in figure 1. Radius of the circle equals standard error of the mean.

then be expressed as the "thiamine intake for equivalent growth" minus the actual thiamine intake. Thus in figure 1, rats on the standard diet receiving penicillin had a mean weight change of 0.9 gm per three weeks between the first and 4th week. From figure 2, the "thiamine intake for equivalent growth" on the standard diet was 1.8 μg per day. From food intake it was calculated that the actual thiamine intake was 0.2 μg per day. Consequently the "thiamine-sparing" of penicillin was equivalent to 1.6 μg of thiamine per day. Similarly, protein had a "sparing" action of 1.8 μg

per day, fat 1.5 μg per day, and ascorbic acid 2.3 and 5.2 μg per day at the low and high levels respectively.

The effect of sparing agents on growth with graded levels of thiamine is shown in figure 2. The sparing effects of protein, penicillin, and ascorbic acid appeared to be independent of thiamine intake, which is to say that the effects of thiamine and these agents on growth were additive. Mameesh et al. ('56) have reported that penicillin is less effective at higher thiamine intakes, but we could not confirm this. Fat was more effective as a sparing agent when higher levels of thiamine were given. This was originally shown by Evans and Lepkovsky ('29).

The effect of combinations of sparing agents is shown in table 2. Antibiotics in combination were no more effective than singly. Fat and ascorbic acid had a considerably greater than additive effect on growth, but in all other cases the effects of sparing agents appeared to be approximately additive. Doubling the concentration of penicillin caused no increase in sparing. In the standard diet, 7.5% of ascorbic acid was no more effective than 5%.

The effects of graded levels of thiamine and of sparing agents on the body content of thiamine are shown in table 3. In all cases, the effects of sparing agents on total body thiamine were reasonably consistent with growth response in comparison with graded intakes of thiamine. There was comparatively little variation in carcass level of thiamine. Differences in the thiamine content of liver corresponded only roughly with differences in total thiamine content. In the case of diets containing penicillin, Guggenheim et al. ('53), using rat liver, have found results similar to ours. The total thiamine content of 10 weanling rats of the same average weight as those started on these experiments was 127 ± 7 μg.

The effect of pyrithiamine on weight change is shown in figure 1. Pyrithiamine depressed growth to approximately the same extent on the standard, fat, and protein diets. By contrast, penicillin and ascorbic acid had little sparing action in the presence of pyrithiamine.

TABLE 2

Combinations of thiamine-sparing agents and their effect on growth

DIET	NO. OF ANIMALS	WEIGHT CHANGE	THIAMINE INTAKE	"THIAMINE INTAKE FOR EQUIVALENT GROWTH"	
				Observed	Calculated as additive
		gm/3 weeks [1]	*μg/day*	*μg/day* [1]	*μg/day* [1]
Standard	36	— 24.1 ± 1.3	0.2	0.4 ± 0.2	
Fat	34	— 0.8 ± 1.6	0.2	1.7 ± 0.2	
Protein	25	8.6 ± 1.2	0.6	2.4 ± 0.2	
Standard plus penicillin	37	1.0 ± 2.0	0.2	1.8 ± 0.2	
Fat plus penicillin	36	17.1 ± 3.2	0.3	3.1 ± 0.3	3.4 ± 0.3
Protein plus penicillin	25	27.2 ± 2.8	0.7	3.9 ± 0.3	4.1 ± 0.3
Standard	14	— 18.0 ± 2.8	0.2	0.7 ± 0.3	
Fat	15	— 1.2 ± 1.7	0.2	1.7 ± 0.3	
Protein	14	8.3 ± 4.2	0.6	2.4 ± 0.4	
Standard plus high-ascorbic acid	15	27.5 ± 12.4	0.3	3.9 ± 1.1	
Fat plus high-ascorbic acid	13	69.8 ± 5.0	0.3	7.6 ± 0.6	5.4 ± 1.1
Protein plus high-ascorbic acid	15	43.4 ± 5.0	0.9	5.2 ± 0.5	6.3 ± 1.2
Standard	10	— 24.3 ± 2.9	0.2	0.4 ± 0.3	
Protein	10	0.6 ± 2.6	0.5	1.8 ± 0.3	
Standard plus low-ascorbic acid	10	— 6.8 ± 4.0	0.2	1.3 ± 0.4	
Standard plus penicillin	10	9.4 ± 3.5	0.3	2.4 ± 0.4	
Protein plus low-ascorbic acid	10	33.2 ± 7.0	0.8	4.4 ± 0.7	3.2 ± 0.5
Standard plus penicillin plus low-ascorbic acid	11	26.4 ± 4.9	0.3	3.8 ± 0.5	3.5 ± 0.6
Standard	14	— 25.9 ± 2.8	0.2	0.3 ± 0.3	
Standard plus penicillin	14	— 2.5 ± 0.9	0.2	1.6 ± 0.1	
Standard plus high-ascorbic acid	15	27.2 ± 6.1	0.3	3.9 ± 0.5	
Standard plus penicillin plus high-ascorbic acid	15	39.5 ± 6.5	0.3	4.9 ± 0.6	5.3 ± 0.6
Standard	14	— 18.6 ± 2.7	0.2	0.7 ± 0.3	
Standard plus penicillin	15	1.6 ± 2.1	0.2	1.8 ± 0.3	
Standard plus streptomycin	15	— 4.1 ± 2.4	0.2	1.5 ± 0.3	
Standard plus penicillin plus streptomycin	14	— 0.8 ± 2.9	0.2	1.7 ± 0.3	3.1 ± 0.4
Standard	10	— 27.3 ± 2.5	0.2	0.2 ± 0.3	
Standard plus penicillin	10	9.5 ± 2.5	0.2	2.5 ± 0.3	
Standard plus aureomycin	10	— 3.7 ± 4.4	0.2	1.5 ± 0.5	
Standard plus penicillin plus aureomycin	10	— 1.1 ± 3.4	0.2	1.7 ± 0.3	3.8 ± 0.5
Standard	10	— 27.3 ± 2.5	0.2	0.2 ± 0.3	
Fat	10	0.1 ± 1.5	0.2	1.8 ± 0.2	
Standard plus low-ascorbic acid	10	10.0 ± 12.5	0.2	2.5 ± 1.2	
Fat plus low-ascorbic acid	10	68.4 ± 10.7	0.4	7.5 ± 1.1	4.3 ± 1.2

[1] Mean and standard error of the mean.

TABLE 3

Thiamine content of animals[1] as affected by graded amounts of thiamine and by sparing agents

DIET	THIAMINE INTAKE[2]		WEIGHT CHANGE[3]	"THIAMINE INTAKE FOR EQUIVALENT GROWTH"[4]	LIVER Thiamine content		CARCASS Thiamine content[5]	
	μg/day	*μg/4 weeks*	*gm/3 weeks*	*μg/day*	*μg/gm*	*μg/animal*	*μg/gm*	*μg/animal*
Standard	0.2	6	— 29.5 ± 1.8	0.1	0.46 ± 0.08	1.8 ± 0.3	0.29 ± 0.02	18.0 ± 1.9
Standard + 2 μg thiamine	2.2	62	0.3 ± 3.5	1.8	0.86 ± 0.08	5.0 ± 0.5	0.38 ± 0.03	33.4 ± 3.6
Standard + 4 μg thiamine	4.3	120	30.8 ± 3.4	4.2	0.82 ± 0.06	6.1 ± 0.5	0.39 ± 0.02	45.0 ± 3.8
Standard + 6 μg thiamine	6.3	176	54.0 ± 2.3	6.1	0.99 ± 0.09	7.6 ± 1.3	0.40 ± 0.03	55.0 ± 6.8
Fat	0.2	6	— 3.4 ± 2.7	1.5	0.26 ± 0.02	1.4 ± 0.1	0.29 ± 0.03	26.1 ± 3.9
Protein	0.5	14	8.1 ± 4.2	2.4	0.50 ± 0.03	2.6 ± 0.2	0.35 ± 0.03	27.8 ± 3.4
Standard + penicillin	0.3	8	25.0 ± 4.4	3.7	0.79 ± 0.10	5.2 ± 0.6	0.36 ± 0.01	39.3 ± 2.8
Standard + high ascorbic acid	0.4	11	71.1 ± 8.4	7.9	1.36 ± 0.12	11.8 ± 1.6	0.54 ± 0.06	74.8 ± 12.0

[1] Mean and standard error of the mean of 10 determinations on each diet.

[2] Thiamine from the diet plus injected thiamine.

[3] Weight at end of 4 weeks minus that after one week on the diets.

[4] Calculated from weight change and from figure 2.

[5] Including liver but excluding skin.

DISCUSSION

Two explanations of the thiamine-sparing action of fat have previously been offered. The first, proposed by Guha ('31), suggested that one of the effects of thiamine deficiency was a pronounced anorexia, which in itself might limit growth. Since a high-fat diet contained more calories per gram than a low-fat diet, fat might exert its effect by allowing an increased caloric intake. If this explanation were correct, fat should be effective in deficiency but should not affect the thiamine requirement. Our results indicate, however, that fat is more effective at higher thiamine levels than at low and thus considerably decreases the thiamine requirement.

The second explanation was a metabolic one, in which it was proposed that fat metabolism did not require the oxidation of pyruvate as a step, while carbohydrate metabolism did. Since thiamine pyrophosphate was a known coenzyme of pyruvate oxidation, the animal might maintain a more nearly normal metabolism on limiting amounts of thiamine by metabolism of fat rather than of carbohydrate. The principal objection to this theory, as pointed out previously (Wright and Scott, '54), is that thiamine pyrophosphate is supposed to be a coenzyme of other oxidations, especially that of α-ketoglutarate, which is an obligatory step in fat oxidation. This objection is not necessarily valid however, since it has been shown that thiamine deprivation limits pyruvate oxidation to a much greater extent than it does α-ketoglutarate oxidation (Wright and Scott, '54).

If the metabolic explanation of thiamine sparing by fat is correct, one would expect the *total thiamine content* (micrograms per animal) of the animal to be the same after a period on a low-fat thiamine-free diet as on a high-fat thiamine-free diet. Since the weight of animals on high-fat diets is often considerably greater than that of those on low-fat diets, the *tissue thiamine level* (micrograms per gram) should be lower. However, animals on high-fat diets, being larger, eat more calories, and on an isocaloric diet will therefore have a higher thiamine intake, if there is any thiamine at all in the diet.

Kemmerer and Steenbock ('33) found that the tissue levels of thiamine in liver and muscle of rats, chicks and pigs were approximately the same on high- or low-fat diets. Evans and Lepkovsky ('35) reported that the tissue levels of thiamine of liver and muscle (but not of brain) of rats were lower in animals on low-fat diets. The animals on high-fat diets weighed almost twice as much as those on low-fat diets; and liver and muscle weights were almost double. Consequently, total thiamine content was markedly higher on high-fat diets. Gruber ('53) reported the tissue levels of thiamine in liver and heart of thiamine-deficient pigeons to be higher on high-fat diets, while brain and breast muscle showed just the opposite. Gershoff and Hegsted ('54) found tissue levels in liver and the carcass of mice to be the same on high and low fat diets. Our results show that, in liver, total thiamine is slightly less, while tissue level is considerably less than on low-fat diets. Total thiamine of carcass including liver, however, is higher in animals on high-fat diets.

These conflicting results are not necessarily difficult to reconcile. It should be noted that tissue level of thiamine is a poor criterion of thiamine status compared to growth. This is partly due to individual variability, which is high, but the crux appears to be that at any level of thiamine intake which limits growth, additional thiamine is used, by and large, for additional tissue and not to increase tissue thiamine level. This is evident from table 3, where levels of thiamine intake from 2.2 to 6.3 μg per day caused little change in carcass thiamine level, although retention of the added injected thiamine was about 20%. It should also be noted that the level of thiamine in liver and presumably, from Gruber's results, in other tissues may not be correlated with total body thiamine.

A third possible explanation is that fat in the diet may alter intestinal flora in much the same way as an antibiotic does. Certainly, the complete substitution of carbohydrate by fat, as in our experiments, could cause a major change in intestinal environment. The effect of fat on the thiamine content of the rat agrees with this concept, although the

increase in content is not quite as large as one would expect from the weight change. This explanation is inconsistent with two facts. First, the effect of fat is additive with penicillin, and thus if both have an effect on intestinal flora, they must act in quite different ways. Second, antibiotics have a general effect on vitamin requirements, while fat does not. Penicillin decreases the requirement for thiamine, riboflavin, pantothenate (Lih and Baumann, '51) and pyridoxine (Sauberlich, '52). A search for similar effects with fat showed that it had no effect on riboflavin and pyridoxine requirements and caused a slight increase in pantothenate requirement (table 4).

The effect of protein on thiamine requirement has been assumed to have the same metabolic basis as the effect of fat. If so, it should be somewhat less effective than fat, since some amino acids are oxidized with pyruvate as an intermediate step. Our results show however, that even if the thiamine intake is considered, protein has more sparing action than fat. Another experiment which is not in accord with this metabolic explanation is that of Kaunitz et al. ('55), in which it was found that thiamine-deficient animals on high-protein diets also receiving pyrithiamine did not live as long as those on carbohydrate diets. We could not confirm these results, since the average survival times on standard, high-fat and high-protein diets of rats (10 per group) receiving 50 μg of pyrithiamine per day were 14.6 ± 0.6, 15.9 ± 0.4 and 14.6 ± 0.5 days respectively. Curves showing growth and weight difference of our animals are given in figure 1. In the presence of pyrithiamine, performance of animals on high-fat diets was superior to that of those on the standard diet, which is in accord with the metabolic explanation of the sparing action of fats. The discrepancy between our results with protein and those of Kaunitz et al. cannot be definitely explained. It is suggested, however, that time of death may be related in part to body weight at the time of death. Animals on high-protein diets show an initial retardation of growth which prevents their attaining a size

TABLE 4

Effect of fat and protein on other vitamin deficiencies as measured by weight gains

VITAMIN	+ VITAMIN	− VITAMIN	+ VITAMIN	− VITAMIN	NO. OF ANIMALS PER GROUP	EXPERIMENTAL PERIOD
	Weight gain in grams[1]					
	gm	*gm*	*gm*	*gm*		*weeks*
	Standard diet		Fat diet			
Riboflavin	110.8 ± 5.0	19.6 ± 3.3	92.1 ± 6.1	13.4 ± 3.7	10	4
Pyridoxine	102.7 ± 6.6	47.6 ± 7.3	96.4 ± 6.2	48.9 ± 7.5	10	4
Pantothenate	98.5 ± 3.8	37.2 ± 4.2	95.0 ± 4.1	21.6 ± 1.9	20	4
Other vitamins[2]	155.5 ± 5.6	143.6 ± 5.2	155.6 ± 5.7	147.6 ± 5.3	20	6
	Standard diet		Protein diet			
Riboflavin	184.4 ± 8.1	22.3 ± 1.9	171.9 ± 8.7	17.4 ± 5.2	15	6
Pyridoxine	199.3 ± 7.1	66.3 ± 4.7	176.9 ± 5.5	60.3 ± 5.5	15	6
Pantothenate		51.4 ± 6.4		85.2 ± 9.7	15	6
Other vitamins[2]	180.9 ± 4.0	174.3 ± 4.1	181.4 ± 6.3	169.9 ± 6.2	15	6

[1] Mean and standard error of the mean.

[2] In the diets lacking other vitamins, niacin, biotin, folic acid, inositol, *p*-aminobenzoic acid, vitamin B_{12} and 2-methylnaphthoquinone were omitted.

comparable to that in the other groups before rapid weight loss begins, and they may be at a disadvantage when rapid weight loss leading to death occurs. In any case, the difference curves in figure 1 for the three diets are indistinguishable, which indicates that the effects of pyrithiamine on weight change are independent of diet composition.

The sparing action of protein could also be an effect on intestinal flora. The arguments for this explanation, and the objections to it, are the same as those stated for the case of fat. The effect of protein differs from that of fat in two respects: it is more effective, but it does not delay significantly the onset of weight loss as does fat. This latter effect could possibly be masked by the poor initial weight gain of rats on high-protein diets.

The effect of penicillin in sparing thiamine has never been considered as other than an effect on intestinal flora, since injected penicillin is ineffective (Guggenheim et al., '53). There appear to be only three ways in which penicillin could spare thiamine, namely, it may favor growth of thiamine-synthesizing flora by elimination of competitive types; it may inhibit bacteria which normally utilize thiamine and thus divert the vitamin from the host; or it may inhibit bacteria which would otherwise have a depressing effect on growth.

That the effect of penicillin is one of inhibition of mildly toxic bacteria which depress growth is unlikely, since one would expect an effect of penicillin on normal animals. This has not been found by others (Lih and Baumann, '51; Sauberlich, '52; Guggenheim et al., '53; Schendel and Johnson, '54) nor by us. Furthermore, as shown in table 3, penicillin actually increases the thiamine content of deficient animals. Penicillin could not decrease diversion of dietary thiamine from host to the flora in sufficient amount to account for the increase in thiamine content found in table 3, and thus this explanation is untenable.

The most serious objection to the postulate that the effect of penicillin is one of favoring bacterial synthesis of thiamine is that one must assume a specificity of penicillin and other

antibiotics with respect to microorganisms such that those organisms which do not show appreciable synthesis of thiamine and other B vitamins are inhibited, while organisms which do show synthesis are not inhibited. There is no independent evidence that this assumed specificity is justified.

Ascorbic acid is not present in sufficient amount in the diet to postulate a metabolic effect as can be done for fat and protein. Furthermore, animals given ascorbic acid have a much higher thiamine content. Ascorbic acid is far more effective than any of the other agents, and 5% in the diet allows a majority of the animals to grow at nearly normal rate on an intake of only 0.4 μg of thiamine per day. At the end of 12 weeks on the standard diet containing 5% of ascorbic acid, all of 10 male rats had survived, and had a mean weight of 241 gm (range 111 to 358 gm). Unlike other sparing agents, there was a great variability in response of individual animals to ascorbic acid. Some animals showed almost normal growth while others barely maintained weight.

Ascorbic acid may have an effect on intestinal flora, but if so, it is different in one respect from that of other antibiotics, namely, the effect of penicillin is not additive with that of other antibiotics, while the effects of penicillin and ascorbic acid are. Ascorbic acid, if it affects intestinal flora, must cause a marked increase in bacterial synthesis of thiamine, and probably of other B vitamins as well (Daft and Schwarz, '52).

In conclusion, no really satisfactory explanation for these sparing agents can as yet be proposed. If it is assumed: (1) that all the effective agents increase bacterial synthesis of thiamine; (2) that penicillin, ascorbic acid and fat or protein affect such synthesis in different ways; and (3) that fat and protein allow a more nearly normal metabolism in thiamine deficiency in addition to their effects on bacterial synthesis, the available data can be fairly well reconciled. Of these three assumptions, only the third is supported by independent evidence.

SUMMARY

The effects of thiamine, fat, protein, penicillin and ascorbic acid, singly and in combination in the diet, on the growth of rats were studied. The effects of these agents on thiamine concentration of tissues were determined.

Fat or protein, penicillin, and ascorbic acid were effective sparing agents in combination. Penicillin plus streptomycin or aureomycin was no more effective than penicillin alone.

All 4 sparing agents increased the thiamine content of the animals in approximate proportion to their effects on growth.

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WEIGHT REDUCTION IN OBESE YOUNG MEN[1]

METABOLIC STUDIES

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A number of workers have reported metabolic studies of obese young women during weight reduction by controlled feeding. (Brown and Ohlson, '46; Brown et al., '46; Leverton and Rhodes, '49; Leverton and Gram, '51; Brewer et al., '52; Cederquist et al., '52; Young, '52a,b; Young et al., '53.) Regardless of the type of diet used, in most instances calcium, nitrogen and phosphorus retentions were less satisfactory during weight reduction than in non-obese women of comparable age or with the same obese subjects in the pre-reduction period. Furthermore, there was a tendency for retentions to diminish as the periods of weight reduction were prolonged. No adequate explanations have been advanced for the poor calcium retentions. In this laboratory (Young, '52a,b; Young et al., '53), studies with obese young women have shown that in the pre-reduction weight-maintenance period all subjects retained calcium, phosphorus and nitrogen. During the first part of the reduction phase (three to 4 weeks), some of the subjects were still retaining and others were just in equilibrium; later in the reduction period (8to 10 weeks) the majority were losing these nutrients. However, after 3.5 weeks on a post-reduction weight-maintenance diet there was almost complete reversal of the downward trend, and

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subjects were in a state of equilibrium or retention with regard to all three nutrients.

No similar investigations have been reported with obese young men. The present study was undertaken, therefore, to study the metabolism of calcium, phosphorus and nitrogen in obese young men of college age (a) in the obese state before weight reduction began, (b) during weight reduction and (c) during a post-reduction weight-maintenance period. A type of diet, namely high-protein, moderate-fat, similar to that employed with the young women was used.

SUBJECTS AND METHODS

A description of the college men used as subjects in the 15-week experiment is given in table 1. The mean and median age of the men was 22 years; the initial weights ranged from 178 to 222 lb with a mean of 198 lb.

The subjects were from 12.2 to 32.8% (mean 18.4%) in excess of ideal weights. Ideal weights were determined on the basis of body build, as estimated by a method devised by Showacre (Moore et al., '55). This method is dependent upon the ratio between internal chest diameter, as measured radiographically, and body height. Using the estimate of body build the ideal weights were read from the Metropolitan Life Insurance Company Tables of Ideal Weights ('43). From the chest x-rays, measurements were also made of subcutaneous fat over the lateral aspect of the thorax at the level of the 10th or 11th rib. Subjects were carefully screened by psychological testing in order to select only those men with sufficient emotional stability to cooperate in controlled feeding over a 15-week period (Summerskill and Darling, '55).

The plan of the experiment included first a 16-day pre-reduction weight-maintenance period followed by a 58-day reduction period which was interrupted between the 33rd and 34th days by a 9-day period of spring vacation during which the men either maintained their weight or lost slightly. Weight reduction was followed by a 30-day period of post-reduction

TABLE 1

Description of subjects and weight changes during experiment

SUBJECT NO.	AGE	HEIGHT	INITIAL WEIGHT	IDEAL WEIGHT	EXCESS WEIGHT		SUBCUTANEOUS FAT PAD [1]	WEIGHT CHANGES				EXCESS WEIGHT LOST	RATE OF WEIGHT REDUCTION
								Pre-reduction (16 days)	Reduction (58 days)	Post-reduction (30 days)	Entire experiment (104 days)		
	yrs.	*in.*	*lb.*	*lb.*	*lb.*	*%*	*mm*	*lb.*	*lb.*	*lb.*	*lb.*	*%*	*lbs./wk.*
1	21	76	221.8	187	34.8	18.6	..	— 2.3	— 22.8	+ 0.3	— 24.8	71.3	2.75
2	22	70	189.6	157	32.6	20.8	10	+ 1.4	— 24.5	+ 0.1	— 23.0	70.6	2.96
3	21	68	188.8	163	25.8	15.8	15	— 1.9	— 27.5	+ 4.5	— 24.9	96.5	3.32
4	26	67	183.4	159	24.4	15.3	20	+ 4.4	— 18.1	+ 1.6	— 12.1	49.6	2.18
5	20	67	220.5	166	54.5	32.8	8	— 3.1	— 25.8	+ 3.4	— 25.5	46.8	3.11
6	22	69.5	177.9	153	24.9	16.3	9	— 1.4	— 13.4	+ 0.5	— 14.3	57.4	1.62
7	22	69	194.0	168	26.0	15.5	16	+ 2.0	— 21.0	+ 0.6	— 18.4	70.8	2.53
8	22	71.5	207.5	185	22.5	12.2	13	— 1.3	— 27.4	+ 0.8	— 27.9	124.0	3.31
Range	20	67	177.9	153	22.5	12.2	8	— 3.1	— 13.4	+ 0.1	— 12.1	46.8	1.62
	to	to	to	to	to	to	to	to	to	to	to	to	to
	26	76	221.8	187	54.5	32.8	20	+ 4.4	— 27.5	+ 4.5	— 27.9	124.0	3.32
Mean	22	69.8	197.9	167	30.7	18.4	13	— 0.3	— 22.6	+ 1.5	— 21.3	73.4	2.72

[1] Lateral aspect of thorax at level of 10th or 11th rib.

weight maintenance. Throughout the experiment the men were weighed by the dietitian weekly on two consecutive days and for two consecutive days at the beginning and the end of each balance period.

The men consumed a weighed diet prepared and served under the supervision of a dietitian at the Special Diet Table operated for research purposes. Only black tea, black coffee or water were allowed ad libitum away from the Table and the quantities of these consumed were carefully recorded. The reduction diet used was of the high-protein, moderate-fat type, similar to that used in previous studies with women in this laboratory, but increased in caloric content to 1800 Cal. Tables 2 and 3 give the distribution of calories supplied by carbohydrate, protein and fat in the basic diets used in the various periods of the experiment and some of the nutrient content of the diets as determined by calculation and analysis. Throughout the experiment the diet was calculated to contain, on a daily basis, 115 gm of protein, 1.0 gm of calcium and 1.5 gm of phosphorus and to be adequate to meet the Recommended Dietary Allowances (National Research Council, '53) for all other nutrients. Calories of the basic diets in the weight-maintenance periods were adjusted by the addition of calories coming largely from combinations of sugar and butter. For each phase of the experiment a series of 7 menus was calculated and they were used in rotation. During the Spring vacation when subjects were away from the campus they were given meal plans to serve as guides and were asked particularly to control the quantities of milk, cheese and meat used.

Four 7-day balance periods were used to study the metabolism of nitrogen, calcium and phosphorus. These periods were as follows: in the second week of pre-reduction weight maintenance; in the 4th and 8th weeks of weight reduction; and in the 4th week of post-reduction weight maintenance.

During the balance periods all food intake was sampled as served. The 7-day composite was ground and mixed to a slurry. Aliquots of the slurry were digested with 2:1 hydro-

chloric acid and saved for analysis. Foods used for additional calories were similarly sampled and analyzed. Collections of feces and nrine were made for the 7 consecutive days of each balance period. Stools were marked by carmine and pooled on a 7-day basis. The nutrient contents of

TABLE 2

Distribution of calories supplied by carbohydrate, protein and fat in the basic diets

PERIOD	TOTAL CALORIES	CARBOHYDRATE		FAT		PROTEIN	
		gm	*% Total calories*	*gm*	*% Total calories*	*gm*	*% Total calories*
I. Pre-reduction maintenance	3800	373	39	210	49	115	12
II. Reduction	1800	104	23	103	51.5	115	25.5
III. Post-reduction maintenance	2800	273	39	139	45	115	16

TABLE 3

Nutrient content of basic diets as determined by calculation and analysis

PERIOD	CALORIES (CALCULATED)	NITROGEN		PHOSPHORUS		CALCIUM	
		Calculated	Analyzed	Calculated	Analyzed	Calculated	Analyzed
	per day	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*
I. Pre-reduction maintenance	3800	18.29	17.97	1.514	1.623	1.059	1.043
II. Reduction	1801	18.45	17.46	1.540	1.493	1.025	1.024
III. Reduction	1801	18.45	17.33	1.540	1.526	1.025	1.096
IV. Post-reduction maintenance	2804	18.44	18.43	1.542	1.603	1.015	1.127

the urine, feces and food aliquots were determined by the following methods: nitrogen, by the Kjeldahl method (Hawk et al., '47); calcium, by the micromethod of Kochakian and Fox ('44); and phosphorus, by the colorimetric method of Koenig and Johnson ('42).

Basal 24-hour caloric requirements of each subject were determined near the beginning of the reduction period (second

week) and again in the second week of the post-reduction period. Each subject spent the night in the Infirmary and basal determinations were made the following morning by a trained technician.[2] The basal metabolic rate was taken as the average of two tests made under suitable basal conditions.

For each balance period, the 17-ketosteroids present in the composite 7-day urine sample of each subject were determined by the method described by Drekter et al. ('47).

RESULTS AND DISCUSSION

Weight loss and caloric needs

Weight changes in the various phases of the experiment are presented in table 1. In the 16-day pre-reduction period on an intake of 3800 Cal. the range in weight change was from — 3.1 to + 4.4 lb. with a mean loss of 0.3 lb. and a median loss of 1.4 lb. For subjects 4 and 7, the caloric level was obviously too high for weight maintenance; for subjects 1, 5 and 8 it was too low and was increased to 4255 Cal. in the last days in order to achieve weight maintenance for these men. The reduction period of 58 days on an intake of 1800 Cal. led to weight losses ranging from 13.4 to 27.5 lb., with a mean loss of 22.6 lb. The rate of weight loss averaged 2.72 lb. per week during the reduction period. In the post-reduction calories led to weight losses ranging from 13.4 to 27.5 lb., with period of 30 days the basal diet was cut back to 2800 Cal. and additional relatively nutrient-free calories were added in appropriate quantities as needed by various subjects to maintain weight. In the relatively short period caloric adjustments were not perfect and weight gains ranging from 0.1 to 4.5 lb. for the 30-day period (mean, 1.5 lb.; median 0.7 lb.) resulted. The average daily caloric intakes which led to these weight changes ranged from 2900 to 3575 with a mean of 3253 and a median of 3375 Cal. per day.

The decrease of caloric needs for weight maintenance in the post-reduction as compared to the pre-reduction period is of interest. In the post-reduction period on a median caloric

[2] Sanborn Metabolator.

intake over 400 Cal. less than in the initial period, the subjects were gaining slightly rather than losing as had been true in the initial period. The observation is in line with previous reports with women (Brown and Ohlson, '46; Young, '52a; and Young et. al., '53) and with rats [3] in which there were diminished caloric needs with weight reduction due, in part, to decreased weight and, in part, to decreased basal metabolism. In young adult rats, it has been shown that the calories required to maintain weights of rats first made obese, then reduced to the pre-obesity weights were less than those required by control animals kept at the pre-obesity weight throughout the experiment.

Weight losses during the reduction period were studied in relationship to various factors. They appeared to be related in a general way to initial weights, the heaviest subjects losing the most weight. Losses did not appear to be related to initial excess weight or to the thickness of the subcutaneous fat pad over the lateral aspect of the thorax at the 10th or 11th rib. These findings are similar to those of Grossman and Sloane ('55) who found that weight loss in normal young men during caloric restriction was correlated with initial body weight, but not with initial body fatness.

Weight losses observed during the reduction period were compared with predicted losses calculated by a method proposed by Jolliffe ('52) based on actual basal caloric requirements plus a 50% additional allowance for activity. The actual losses exceed the predicted losses in every case, the range being from 105.4 to 192.3%, the mean and median both being 156%. It seems likely that the college men were more active than those for whom the activity figures were designed. Subject 3 was the most active of the men and his actual loss clearly exceeded the prediction by the greatest amount.

In examining individual weight graphs it was interesting to confirm previous observations made in this laboratory with the use of the high-protein, moderate-fat diet, that, in general,

[3] Young, C. M., and V. U. Serraon, unpublished data.

weight is lost at a fairly regular and continuous rate when food intake is known to be controlled. After an initially more rapid loss, throughout the reduction period there is little or no evidence of "plateauing" except in one subject. Immediately after going on a moderate-fat reduction diet, there is usually an initial sharp drop in weight; return to a higher carbohydrate intake is followed by a sudden increase in weight unrelated to the change in caloric intake (Fryer et al., '55; Ohlson et al., '55; Kekwick and Pawan, '53).

METABOLIC STUDIES

A summary of the mean daily retentions of nitrogen, calcium and phosphorus in the 4 balance periods is presented in table 4. As in previous reports, the practice was followed of, in the case of nitrogen, considering only amounts greater that 5% of intake as retentions or losses, since it is difficult to be certain of the accuracy of the demarcation of carmine-labelled feces. In the case of calcium and phosphorus, 10% of intake marks the boundaries of equilibrium since a much larger proportion of the excretion of these nutrients appears in the feces than is the case with nitrogen.

During the pre-reduction and post-reduction periods of "intended" weight maintenance, all subjects were in equilibrium or were retaining all three nutrients regardless of whether or not they were in absolute weight maintenance. During the reduction period on the 1800 Cal. level, losses of one or more nutrients occurred in 5 of 8 subjects; however, three subjects (2, 4 and 7) did not go into negative balance for any of the nutrients during the entire experiment.

Nitrogen metabolism. In the balance study during the pre-reduction period, on a mean intake of 17.976 gm of nitrogen per day, 7 of the 8 men were retaining nitrogen; the 8th was in equilibrium. In the second balance period, at the 4th week of caloric restriction, 4 subjects were losing nitrogen and 4 were in equilibrium. By the 8th week of reduction the same 4 subjects were continuing to lose nitrogen; one of the 4 who had been in equilibrium was retaining nitrogen. In

TABLE 4

Mean daily retentions of nitrogen, calcium and phosphorus

BALANCE PERIOD	RETENTION BY SUBJECTS								MEAN INTAKE	SUMMARY		
	1	2	3	4	5	6	7	8		Number of subjects		
										R [1]	B [2]	L [3]
	gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*			
Nitrogen												
I. Pre-reduction, second week	+ 2.197R [1]	+ 2.714R	+ 2.082R	+ 3.221R	— 0.073B [2]	+ 2.505R	+ 2.301R	+ 2.304R	17.976	7	1	0
II. Reduction, 4th week	— 2.172L [3]	— 0.795B	— 1.147L	— 0.212B	— 1.362L	— 1.130L	— 0.017B	+ 0.273B	17.459	0	4	4
III. Reduction, 8th week	— 1.464L	+ 1.014R	— 0.930L	+ 0.580B	— 1.144L	— 1.589L	— 0.454B	— 0.574B	17.330	1	3	4
IV. Post-reduction, 4th week	+ 0.036B	+ 0.807B	+ 1.458R	+ 1.036R	— 0.855B	— 0.011B	+ 0.216B	+ 0.405B	18.467	2	6	0
Calcium												
I. Pre-reduction, second week	— 0.100B	+ 0.056B	— 0.043B	— 0.094B	— 0.030B	— 0.043B	— 0.077B	— 0.103B	1.049	0	8	0
II. Reduction, 4th week	— 0.127L	+ 0.067B	— 0.170L	+ 0.003B	— 0.015B	+ 0.243R	— 0.057B	— 0.132L	1.054	1	4	3
III. Reduction, 8th week	— 0.066B	+ 0.105B	+ 0.043B	+ 0.133R	+ 0.157R	+ 0.222R	+ 0.070B	+ 0.081B	1.136	3	5	0
IV. Post-reduction, 4th week	— 0.027B	+ 0.151R	+ 0.173R	+ 0.270R	+ 0.168R	— 0.088B	+ 0.143R	+ 0.046B	1.202	5	3	0
Phosphorus												
I. Pre-reduction, second week	+ 0.062B	+ 0.167R	+ 0.137B	+ 0.412R	+ 0.042B	+ 0.261R	+ 0.200R	+ 0.143B	1.624	4	4	0
II. Reduction, 4th week	— 0.251L	+ 0.109B	— 0.153L	— 0.087B	— 0.213L	+ 0.007B	+ 0.002B	— 0.153L	1.493	0	4	4
III. Reduction, 8th week	— 0.263L	+ 0.029B	— 0.171L	— 0.024B	— 0.164L	— 0.037B	— 0.074B	— 0.120B	1.526	0	5	3
IV. Post-reduction, 4th week	— 0.037B	+ 0.213R	+ 0.174R	+ 0.083B	— 0.055B	— 0.097B	+ 0.064B	+ 0.009B	1.611	2	6	0

[1] R = Retention greater than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

[2] B = Balance: retention or loss less than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

[3] L = Loss greater than 5% of intake for nitrogen or 10% of intake for calcium and phosphorus.

contrast to the findings previously reported with women (Young, '52b; Young et al., '53), the differences between the retentions of individuals in the two balance periods during weight reduction were not of sufficient magnitude nor were they consistent enough to indicate any trend toward better or poorer retention as the period of caloric restriction lengthened. In the 4th week of post-reduction weight maintenance, all subjects were either in balance or retaining nitrogen. Thus, in both periods when caloric intake was either adequate, or nearly adequate to maintain weight, no subject was losing nitrogen.

Nitrogen retentions of individuals were examined in relation to the following: total weight losses, total weights lost by the end of each balance period, initial weights of subjects, initial excess weights and changes in basal metabolism from early reduction to post-reduction maintenance. A direct relationship between nitrogen retentions and any one of these factors was not apparent.

Urinary and fecal fractions of the nitrogen excreted during various balance periods were examined and expressed as percentages of intake. When the caloric intake dropped from 3800 or more calories to 1800 two things happened. Urinary excretions of nitrogen increased from a mean of 77.86% to a mean of 98.61% of intake at the 4th week of reduction and to a mean of 97.10% at the 8th week. Thus there was a sharp increase in the urinary nitrogen excretion expressed as percentage of intake; the increase was shown by every subject studied. Fecal nitrogen decreased from a mean of 10.01% to means of 6.04 and 6.32% of intake at the 4th and 8th week respectively. Here again, every individual showed the drop.

When calories were increased to a level adequate for weight maintenance, the percentage of intake excreted as urinary nitrogen decreased somewhat (90.45) and the percentage excreted as fecal nitrogen increased (7.46). These trends were shown by the group as a whole and by every individual. However, the figures did not return, by the 4th week of post-

reduction weight maintenance, to the pre-reduction levels. No adequate explanation is available for the greater excretion of nitrogen in the urine in the post-reduction weight maintenance period as compared with the pre-reduction period.

Hegsted et al. ('46) have suggested that the amount of fecal nitrogen is dependent on the amount of food consumed, that is, it increases with increase in total food intake. In the present study, the data suggest that fecal nitrogen might be related to the amount of food consumed. Both amounts of food consumed and of nitrogen excreted ranked as follows: largest amount in the pre-reduction period, second largest in the post-reduction period, and smallest amount during weight reduction.

Nitrogen loss per kilogram of body weight lost during the 4th and 8th weeks of weight reduction was studied in relationship to the only measurement of body fatness available, namely, the measurement made at the beginning of the experiment of the fat pads over the lateral aspect of the thorax, at the 10th and 11th rib. There was great variation among the subjects in the amount of nitrogen lost per kilogram of body weight lost. In the early reduction balance period the 4 men who showed the least nitrogen loss relative to weight loss were those men among the group who had had the thicker subcutaneous fat measurements; in the late reduction balance period three of the subjects who lost the least nitrogen relative to body weight loss were among those who originally had the thickest subcutaneous fat measurements. Conclusions cannot be drawn since the one available measurement of body fat probably was not a true measurement of total body fatness. However it is apparent that as Ohlson et al. ('55) have suggested, one of the basic needs for research in weight reduction is in the body composition of the overweight as it relates to the metabolic responses observed.

Calcium metabolism. All subjects were in calcium equilibrium in the pre-reduction balance period on a mean intake of 1.049 gm of calcium (table 4). In the 4th week of weight reduction, on a mean intake of 1.054 gm, three subjects were

losing calcium in excess of 10% of intake; 4 were in equilibrium and one was retaining. By the 8th week of reduction, on a mean intake of 1.136 gm, the three who had been losing calcium were back in equilibrium, and of the 4 who had been in balance, two were still in balance and two were storing calcium. Thus by late in the reduction phase of the study, 5 subjects were storing, three were in equilibrium, and none was losing calcium.

During the 4th week on the post-reduction weight-maintenance diet, retentions were as good as, or better than, they had been late in the reduction period, with the single exception of subject 6, who dropped from retention to equilibrium. Five subjects were retaining calcium; three were in equilibrium.

The patterns of calcium retentions of the men throughout the experiment are in sharp contrast to those previously observed with the young women (Young, '52 and Young et al., '53). The young women stored calcium in the pre-reduction periods; in the majority of cases they were in equilibrium early in weight reduction but were in calcium deficit by the 8th week of weight reduction. Release of caloric restriction in the post-reduction maintenance period resulted in calcium retention or equilibrium. For the men, calcium retentions improved in the group as a whole as weight reduction was prolonged. The only calcium losses were found in the early reduction period for three subjects.

An examination of the urinary excretions of calcium expressed as percentage of intake in the 4 periods revealed no consistent pattern from period to period. However, in the case of fecal excretion, for 7 of the 8 subjects the percentages of calcium intake excreted in the feces decreased progressively from the first to the 4th balance study with the exception of the three subjects who lost calcium in the early reduction period. For these three men fecal calcium increased in the early reduction period and then continued its downward trend.

We can offer no explanation for the differences in the calcium responses of the men and the women or actually for

the negative calcium balances observed with either group. For the women, we felt the negative calcium balances with prolongation of weight reduction were related to emotional strain with the continued caloric restriction and stress of a controlled feeding regimen. Since the publication of these earlier papers two reports (Stearns, '55; Malm, '55) have lent further weight to the effects of emotional strain as a possible explanation. Both Stearns and Malm made studies of calcium balances of individuals who were not losing weight but who were in such circumstances that emotional states would be expected to fluctuate and that the fluctuations would be apparent to the observer. Both investigators reported that calcium balances were correlated with the emotional status of the individual. The calcium retentions varied inversely with the emotional stress of the subjects.

It will be recalled that in the present experiment an attempt was made to select as subjects men who might be expected to maintain emotional stability under conditions of dietary restriction. Judging from their overt behavior during the experiment the subjects had been correctly appraised at the time of selection. If our appraisal of emotional stability was correct, and if, in fact, a correlation does exist between emotional stability and calcium balances, one would expect to see little change in the calcium metabolism of these subjects. Such proved to be the case. If emotional strain were a factor it would seem that it affected only three of the 8 subjects early in the reduction program and that later in the process the three had become adjusted to the restrictions imposed. The women subjects studied had not been similarly screened on an emotional basis. They did show greater signs of emotional instability during the course of the reduction process.

One further observation can be made concerning the three men who were losing calcium in the balance period at the 4th week of weight reduction. An examination of weight loss records revealed that these three subjects were the three who lost the most weight during the week of the balance period

and who, by the end of the balance period, had lost the greatest percentages of their excess weights. Their weight losses during this week were greater than the weight losses of any subject during the balance period at the 8th week of reduction. Thus the only three subjects who lost calcium at any time during the experiment, also lost, simultaneously, the most weight in any week when balance studies were being made.

It will be recalled that in the pre-reduction balance period all men were in calcium equilibrium; none was retaining calcium. The equilibrium is in contrast to the retention found in similar circumstances with the women. It will be observed however, in data from table 4, that in one or more of the subsequent balance periods 6 of the 8 men showed they could retain calcium on an intake of 1.0 gm. The calcium equilibrium found in the first balance period might be due to the fact that pre-experimental calcium intakes could have been on a higher level. Unfortunately no information is available on intakes previous to the experimental period. However, earlier studies of food intakes of men of the same age range on the campus have shown calcium intakes ranging from 0.743 to 2.433 gm per day, with a mean of 1.499 and a median of 1.536 gm.[4] From these figures one might infer that the habitual calcium intakes of at least some of our subjects might have been higher than the 1.0 gm supplied by the experimental diet. The literature contains little to indicate the effect on retentions of reducing intake from a level higher than one gram to a level of one gram. What evidence there is at other levels is conflicting (Steggerda and Mitchell, '39, '46; Ohlson, '55; Leichsenring et al., '51; Johnston et al., '56). As far as the present study is concerned it would seem that two possibilities should not be ruled out. It is possible that intakes of calcium of at least some subjects were lower than they had been before the experiment began; and that, initially, retentions may have been lower under this influence and have subsequently increased as subjects became adapted to the new intake level.

[4] Young, C. M., unpublished data.

Phosphorus metabolism. In the pre-reduction period 4 subjects were in equilibrium and 4 were retaining phosphorus (table 4). At the 4th week of reduction the 4 who had been in balance were losing phosphorus and the 4 who had been retaining were in equilibrium. By the 8th week of reduction one of the 4 who had lost phosphorus at the 4th week was now in balance while the other three were still losing; the 4 who had been in balance were still in balance. By the 4th week of post-reduction maintenance, all subjects were back in equilibrium or were retaining phosphorus.

Thus, as in the case of nitrogen, all subjects were either in balance or were retaining phosphorus when caloric intakes were approximately adequate for weight maintenance, but 4 of 8 subjects lost phosphorus when calories were inadequate. There was no real evidence of improvement or deterioration with the lengthening of the reduction period. Three of the 4 subjects (1, 3 and 8), who lost phosphorus, were ones who also lost calcium in the early reduction period. Subjects 1, 3 and 5 lost phosphorus and nitrogen in both balance studies during caloric restriction.

The urinary excretion of phosphorus expressed as a percentage of intake, increased in every subject during the periods of caloric restriction. The mean excretions in the 4 successive balance periods, expressed as percentage of intake, were 63.92, 75.70, 75.79, and 70.56 respectively. Though the mean percentage excretion dropped in the post-reduction period, it did not reach the pre-reduction level.

The fecal excretion of phosphorus, similarly expressed, in all but one subject increased in the periods of caloric restriction and then in the post-reduction period returned to nearly the pre-reduction maintenance level. The mean percentages for the first through the 4th balance periods were: 25.11, 30.48, 30.96 and 26.70 respectively.

Basal metabolism. Comparison of the basal metabolic requirements of subjects early in the weight reduction and post-reduction periods are presented in table 5. In 5 of the 8 subjects basal metabolism requirements decreased with

weight reduction by approximately 200 Cal. or more. Two of the subjects showed very little change. The mean decrease was 150 Cal. or 8.4%. The mean percentage decrease is similar to those reported in the previous two studies with women, namely 7.6 and 8.6 respectively (Young, '52b and Young et al., '53). In a general way the absolute decrease in basal metabolism seemed to be related to the total number of pounds lost. With one exception, subject 2, those having the greater weight losses had greater decreases in metabolism than did those with lesser losses.

TABLE 5

Basal metabolism in reducing and post-reducing periods

SUBJECT	WEIGHT LOSS IN REDUCTION PERIOD	BASAL CALORIES PER 24 HOURS		CHANGE IN BASAL METABOLISM	
		2nd week reduction	2nd week post-reduction		
	lbs.			*Cal.*	*%*
1	22.8	2099	1869	— 230	— 11.0
2	24.5	1730	1647	— 83	— 4.8
3	27.5	1796	1539	— 257	— 14.3
4	18.1	1685	1668	— 17	— 1.0
5	25.8	1953	1734	— 219	— 11.2
6	13.8	1633	1664	31	1.9
7	21.0	1813	1577	— 236	— 13.0
8	27.4	1925	1734	— 190	— 9.9
Mean	22.6	1829	1679	— 150	— 8.4

Keto-steroids

The average daily urinary excretions of 17-ketosteroids for each of the 4 balance periods are given in table 6. Keto-steroids were determined as a possible measure of any stress during weight reduction. In general, the results fall within the range of values found by Drekter et al. ('47) for young men between the ages of 20 and 30. These authors found amounts ranging from 10.0 to 28.9 mg with a mode falling between 16 and 17 mg. Drekter et al. report finding between 10 and 20 mg in 79% of the 24-hour urine specimens assayed. In the present study only 8 of the 32 samples assayed con-

tained amounts between 10 and 20 mg. Also, the average values for each of the 4 balance periods are somewhat higher than the average value of 16.9 mg found by Drekter et al. ('47). However, as a measure of possible stress due to caloric restriction the values appear to reveal nothing, since, if anything, urinary 17-ketosteroid excretion is slightly lower during caloric restriction than during caloric adequacy.

TABLE 6

Urinary 17-ketosteroids

SUBJECT NO.	BALANCE PERIOD			
	2nd week pre-reduction maintenance	4th week of reduction	8th week of reduction	4th week of post-reduction maintenance
	mg/24 hrs.	*mg/24 hrs.*	*mg/24 hrs.*	*mg/24 hrs.*
1	31.5	25.2	27.7	32.0
2	25.1	21.6	21.4	24.6
3	19.9	19.5	17.1	27.9
4	22.9	20.0	19.8	22.0
5	24.9	21.3	26.6	26.7
6	25.5	23.2	25.8	27.0
7	21.5	18.3	22.2	21.4
8	24.5	19.8	18.6	22.1
Mean	24.5	21.1	22.4	25.5

SUMMARY

Nitrogen, calcium and phosphorus metabolism, on a constant intake of the three nutrients, was studied in 8 obese young men by means of 7-day balance studies in a pre-reduction period of weight maintenance, at the 4th and 8th weeks of weight reduction and at the 4th week of post-reduction weight maintenance.

Weight losses in the 8-week reduction period ranged from 13.4 to 27.5 lb. with a mean of 22.6 and a median of 23.6 lb. Weight losses appeared to be related to initial body weights.

Four of the 8 subjects went from nitrogen and phosphorus retention or equilibrium in the pre-reduction period to nitrogen and phosphorus loss during the period of caloric restriction. Within the limits of the experiment the losses did

not appear to increase with prolongation of the reduction period. Relief of caloric restriction was accompanied by return to equilibrium or retention with respect to these nutrients but at a lower level.

In contrast to women subjects studied previously, only three of the 8 men went into calcium deficit and the deficit occurred early in the weight reduction period with subsequent return to calcium equilibrium in the later stages of reduction. No satisfactory explanation is given for the changes in calcium retentions; they are discussed in terms of the emotional stability and relative rate of weight loss in the subjects.

In 7 of the 8 subjects 24-hour basal caloric requirements decreased during weight reduction; the mean decrease was 150 Cal. or 8.4%.

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INFLUENCE OF ANTIBIOTICS AND TRYPTOPHAN DEFICIENCY ON GROWTH IN THE RAT [1]

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Previous work (Waisman and Cravioto, '52) on the aminopterin-induced folic acid deficiency in rats, failed to demonstrate any consistently favorable influence on growth when antibiotics were included in the diet. The addition of citrovorum factor to this diet caused a definite response in growth, which was further increased by supplementation with aureomycin. Other workers (see Jones and Baumann, '55 for references) have demonstrated that poor growth resulting from deficiencies of some of the B vitamins could be corrected when certain antibiotics were added. It was of interest to determine whether the antibiotics could act similarly in promoting growth when rats were fed a diet partially deficient in an amino acid.

The influence of antibiotics on amino acid deficiencies induced by the use of amino acid analogues or by amino acid-supplemented low-casein diets has been under investigation in this laboratory for several years. In the experiments reported here, growth responses to various antibiotics were evaluated in typtophan-deficient rats. The experiments were designed to determine: (1) whether the various antibiotics were able to promote growth in rats fed a tryptophan-deficient diet, (2) whether there is a difference in growth response between the male and the female animals, (3) whether there is an addi-

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tional growth effect when an antibiotic is fed simultaneously with an addition of tryptophan to the basal diet, and (4) whether restriction of food intake has any influence on the effect of the antibiotics.

EXPERIMENTAL

Weanling albino rats, of the Sprague-Dawley strain, weighing 40 to 50 gm, were used in these experiments. The composition of the basal ration used throughout was: casein, 9%; DL-threonine, 0.5%; L-histidine, 0.2%; DL-methionine, 0.5%; corn oil, 5%; salts XIV, 4%; vitamins, in milligrams per 100 gm of diet, as follows: thiamine hydrochloride 0.5, riboflavin 1.0, niacin 1.0, pyridoxine 0.5, calcium pantothenate 2.5, biotin 0.02, *p*-aminobenzoic acid 30, i-inositol 100, folic acid 0.02, choline chloride 200, vitamin B_{12} 0.002, Menadione 0.5. Sucrose was added to make 100%. When supplemented, antibiotics were given at a level of 0.01% of the diet. DL-Tryptophan was fed at a level of 0.2% at the expense of the sucrose. The rats were given two drops of oleum-percomorphum, directly into the mouth, weekly, and were housed in screen-bottom individual cages. At least minimal levels of amino acids were added to the 9% casein basal diet to conform to the amounts recommended by Rose ('37). All rats were weighed twice weekly and fed their experimental diets for three weeks.

RESULTS

The data are presented in tables 1 and 2. It was found repeatedly that terramycin, achromycin, and erythromycin, included at a level of 0.01% of the diet, were able to effect a significant increase in growth of male rats, an average of 7 gm per week over that obtained with the 9% casein diet supplemented with amino acids. Chloromycetin, dihydrostreptomycin, bacitracin, polymyxin were without effect on the growth of male rats, while penicillin was borderline.

Terramycin, achromycin and erythromycin were tested with female rats. There seems to be a slight growth increase in

TABLE 1

The effect of antibiotics on rat growth

GROUP	DIET	SERIES I ♂	SERIES II ♂	SERIES III ♂	SERIES III ♀	SERIES IV ♂	SERIES IV ♀	SERIES V ♂
		gm/wk.[1]	*gm/wk.*	*gm/wk.*	*gm/wk.*	*gm/wk.*	*gm/wk.*	*gm/wk.*
1	9% casein + methionine, threonine, histidine	17.7 ± 0.8 (7)[2]	23.2 ± 1.3 (6)	15.1 ± 1.8 (5)	21.2 ± 1.3 (4)	16.2 ± 2.9 (6)	18.2 ± 0.8 (6)	15.1 ± 1.9 (6)
2	(1) + tryptophan	26.7 ± 1.0 (6)	28.3 ± 1.3 (6)	22.0 ± 0.7 (5)	23.8 ± 1.2 (4)	24.9 ± 1.1 (6)	22.7 ± 0.9 (6)	25.7 ± 1.7 (6)
3	(1) + terramycin		27.4 ± 0.7 (6)	23.8 ± 2.0 (5)	22.5 ± 1.1 (4)		20.2 ± 1.2 (6)	25.3 ± 0.8 (6)
4	(2) + terramycin						23.3 ± 0.9 (6)	24.9 ± 0.9 (6)
5	(1) + achromycin	24.4 ± 0.7 (7)	28.4 ± 0.8 (6)	22.0 ± 1.0 (5)	23.2 ± 1.0 (4)		23.4 ± 2.4 (6)	23.1 ± 1.0 (6)
6	(2) + achromycin							25.3 ± 1.8 (6)
7	(1) + erythromycin		27.7 ± 1.1 (6)	24.7 ± 0.9 (5)	20.5 ± 2.4 (4)		21.6 ± 1.5 (6)	27.6 ± 0.8 (6)
8	(2) + erythromycin							24.3 ± 1.5 (6)
9	25% casein	40.1 ± 0.9 (6)		38.1 ± 0.4 (5)	31.4 ± 1.3 (4)			
10	(9) + achromycin	42.3 ± 0.8 (6)						
11	(1) + chloromycetin		22.0 ± 1.2 (6)					
12	(1) + dihydro-streptomycin		24.7 ± 1.0 (6)					
13	(1) + bacitracin		25.2 ± 0.7 (6)					
14	(1) + polymyxin B		24.4 ± 1.0 (6)					
15	(1) + penicillin G		25.9 ± 1.2 (6)					

[1] The mean ± the standard error of the mean.
[2] The number within the parentheses indicates the number of rats used for that group.

TABLE 2

The effect of antibiotics on rat growth when food intake was restricted

GROUP	DIET	SERIES VI				SERIES VII	
		♂ Ad lib.	♂ Pair fed	♀ Ad lib.	♀ Pair fed	♂ Ad lib.	♂ Pair fed
		gm/wk.[1]	*gm/wk.*	*gm/wk.*	*gm/wk.*	*gm/wk.*	*gm/wk.*
1	9% casein + methionine, threonine, histidine	17.6 ± 1.3 (4)[2]		18.1 ± 1.6 (4)		9.1 ± 1.2 (6)	
2	(1) + tryptophan "pair fed" to (1)		19.3 ± 1.1 (4)		20.4 ± 1.4 (4)	25.6 ± 0.6 (6)	
3	(1) + terramycin "pair fed" to (1)		18.8 ± 1.6 (4)		17.9 ± 1.4 (4)		
4	(1) + achromycin "pair fed" to (1)		18.3 ± 1.1 (4)		19.4 ± 1.6 (4)		
5	(2) + achromycin "pair fed" to (2)						25.4 ± 0.9 (6)
6	(1) + chloromycetin "pair fed" to (1)		16.4 ± 0.9 (4)		19.5 ± 2.2 (4)		
7	(1) + erythromycin "pair fed" to (1)	25.8 ± 0.7 (4)	19.1 ± 1.3 (4)	21.2 ± 0.8 (4)	20.0 ± 1.2 (4)		
8	25% casein	42.8 ± 1.3 (4)	26.1 ± 1.9 (4)	28.3 ± 0.9 (4)	24.8 ± 1.1 (4)	38.6 ± 2.1 (6)	
9	25% casein + achromycin "pair fed" to (8)						39.4 ± 1.3 (6)

[1] The mean ± the standard error of the mean.

[2] The number within the parentheses indicates the number of rats used for that group.

the females of series IV, but definitely lower than that obtained with males from series II, III and V. In series III, the lack of growth response may have been masked by the unusually good weight attained by the control group.

The effect of antibiotics fed with or without tryptophan supplementation is shown in the last column in table 1, series V. As can be seen, there were no significant differences among the growth rates obtained when either tryptophan, or antibiotic was fed alone, or when both tryptophan and an antibiotic were fed simultaneously.

Animals receiving antibiotic supplementation were "pair fed" to the food intake of those fed the 9% casein basal to study the effects of restricted food intake. No stimulation of growth was observed under these conditions in rats fed individual antibiotics (table 2).

DISCUSSION

The antibiotics, terramycin, achromycin, and erythromycin, improved growth of male rats fed the 9% casein basal diet. This growth was comparable to that observed in rats receiving the basal diet plus 0.2% of DL-tryptophan. When each of these antibiotics was added to the 9% casein basal diet, supplemented with 0.2% of DL-tryptophan, no further growth response was produced. The good growth obtained on the 25% casein diet was not improved when achromycin was added.

The growth-promoting effect is evidently present only when the supply of one amino acid is suboptimum. When the protein level is optimum, such as in a 25% casein diet, or even when the protein level is low but the amino acid constituents are in balance, such as in a 9% casein diet supplemented with appropriate amino acids, the growth-promoting effect of antibiotics is absent. A similar effect was observed by Sauberlich ('54) with peanut meal and with casein. This effect of antibiotics is probably not specific for one particular amino acid.

Studies on growth responses obtained on diets supplemented with antibiotics have provided many theories to explain the mechanism of this sparing action of the antibiotics. Evidence

has been presented by many authors that the antibiotics act by altering the intestinal flora of the host so that the synthesis of amino acids is favored. There is also the possibility that thinning of the intestinal wall under the influence of antibiotics facilitates absorption of nutrients (Coates et al., '55). Furthermore, Francois and Michel ('55); Michel and Francois ('55) have correlated the effect of penicillin and aureomycin on growth with the ability of the antibiotics to suppress bacterial deaminase activity so that better utilization of amino nitrogen is possible.

We found in our experiments that when food was restricted, none of the antibiotics showed any growth effect (table 2). It is only when the diet is fed ad libitum and intake increases over that of the control that a growth increase is apparent. The lack of growth response in those animals given a restricted intake of suboptimal protein may be due to the inability of the body to respond favorably when both calories and protein are limited. The stimulus to growth by the antibiotics is most readily shown under conditions where all food constituents are optimum and used most advantageously. In restricted food intakes the influence of the antibiotics is obscured. Previous work by Slinger et al. ('54) showed similar finding in chicks using restricted food intake.

The lack of growth response in female rats fed antibiotics is not well understood but may be masked by the slightly greater growth observed in female controls when compared to the males.

SUMMARY

The antibiotics, terramycin, achromycin and erythromycin, when added at a level of 0.01% of the diet, were able to improve the growth of male rats fed a 9% casein diet. When tryptophan was supplied in addition to antibiotics, no further improvement was obtained. The same antibiotics were less effective in female rats.

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