THE CHICK'S REQUIREMENT FOR FOLIC ACID IN THE UTILIZATION OF CHOLINE AND ITS PRECURSORS BETAINE AND METHYLAMINOETHANOL [1]

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INTRODUCTION

Daniel, Farmer and Norris ('46) found that in addition to manganese, choline and biotin, the chick also requires folic acid for the prevention of perosis. Later, Schaefer, Salmon, Strength and Copeland ('50) reported that the chick's requirement for choline is reduced by the addition of folic acid as well as by vitamin B_{12}. They found that a level of 0.6% choline Cl plus vitamin B_{12} was ineffective in the prevention of perosis unless folic acid was also supplied. However, in the presence of both folic acid and vitamin B_{12}, the level of 0.1% choline Cl completely protected the chicks from perosis.

Dinning, Keith and Day ('51) reported that livers from folic acid-deficient chicks have a reduced ability to form methionine from homocystine or homocysteine plus choline or betaine.

In the course of work designed to study the metabolism of choline and its precursors the following experiments were conducted to determine the *in vivo* relationship of folic acid

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and choline in the chick. In particular, it was desired to see if folic acid is required in the formation of choline from betaine and dimethylaminoethanol or monomethylaminoethanol.

EXPERIMENTAL

Cross-bred (RIR × BPR) male day-old chicks obtained from a local hatchery were used in this study. At the beginning of each experiment, the chicks were randomly distributed into lots of 20 chicks per treatment. The chicks were maintained in electrically heated battery brooders with wire-mesh floors. Feed and water were supplied ad libitum. Individual chicks were weighed at weekly intervals. Scoring for perosis was done at the third and 4th weeks.

The basal diet fed the chicks was composed, in per cent, of purified casein 16.5,[3] alcohol-extracted peanut meal 10.0,[3] cornstarch 62.82, hydrogenated fat 2.5, ground cellophane 3.0, iodized salt 0.6, dicalcium phosphate 2.0, limestone 1.0, cystine 0.1, glycine 0.66, arginine HCl 0.061 and minerals.[3] Vitamin supplements in milligrams per kilogram of diet were as follows: alpha tocopheryl acetate 55.0, niacin 26.4, calcium pantothenate 16.5, riboflavin 5.5, pyridoxine HCl 5.5, thiamine HCl 3.3, menadione 2.2 and biotin 0.15. Vitamin B_{12} was added to the diet at a level of 3.0 μg per kilogram. Vitamin A palmitate and irradiated animal sterols in cottonseed oil were added to the ration to supply 2000 I.U. of vitamin A and 135 I.C.U. vitamin D_3 per pound of feed.

The alcohol-extracted peanut meal and purified casein were each assayed for choline by the reineckate method of Glick ('44) and for folic acid by a modification of the method of Daniel, Scott, Norris and Heuser ('45) with *Streptococcus faecalis R.* The folic acid method was modified by hydrolyzing the samples with chick pancreas (Laskowski, Mims and Day, '45) and hog kidney (Bird et al., '45) with the latter hydrolysis done in the presence of cysteine (Hill and Scott, '51). The diet was found to contain 16 μg of folic acid

[3] Young, Gillis and Norris. J. Nutrition, 1953, *50:* 291.

per 100 gm and less than 0.007% choline. Crystalline choline chloride and betaine monohydrate were used as the sources of choline and betaine. Monomethylaminoethanol and dimethylaminoethanol were in the anhydrous form. All supplements, including folic acid, were added to the diet in water solutions which were freshly prepared for each experiment.

RESULTS

The first experiment was designed to obtain a more quantitative measure of the interrelationship of folic acid and choline. The chicks were fed the basal diet except that a level

TABLE 1

The chick's requirement for folic acid at various levels of choline Cl

AM'T. FOLIC ACID PER 100 GM DIET	AMOUNT OF ADDED CHOLINE Cl					
	None		0.05%		0.2%	
	Wt.	Perosis [1]	Wt.	Perosis	Wt.	Perosis
	gm	*%*	*gm*	*%*	*gm*	*%*
None	135±6.30 [2]	100 (19) [3]	219±5.24	75 (19)	243±10.95	80 (19)
30 μg	197±8.92	95 (19)	321±8.61	10 (20)	303± 8.35	0 (20)
80 μg	224±8.71	85 (19)	321±7.11	10 (20)	307±11.25	0 (20)
300 μg	224±7.32	90 (20)	325±9.47	15 (20)	325±11.54	10 (19)

[1] Per cent perosis is based on surviving chicks.

[2] Mean ± standard error.

[3] The numbers in parentheses indicate survivors out of 20 chicks started.

of 0.17% DL-methionine was used in this experiment instead of 0.1% cystine. The results of this experiment are reported in table 1. These results are the average of duplicate lots of 10 chicks per lot, or 20 chicks per treatment.

In the absence of choline chloride, the chick's requirement for supplementary folic acid was not more than 80 μg per 100 gm of diet. However, when 0.05% or more of choline chloride was added to the diet, the folic acid requirement was satisfied with the addition of 30 μg of folic acid per 100 gm of diet. The 0.05% level of choline chloride appeared to be adequate for maximum growth and almost completely pro-

tected this strain of chicks from perosis. In the absence of folic acid, the 0.2% level of choline chloride gave a small but probably significant growth response over the 0.05% level of choline chloride, but failed to protect the chicks from perosis.

Based on the results of this experiment and other preliminary experiments with this strain of chicks, a folic acid level of 80 μg per 100 gm of diet and a level of choline or its precursors at a molar concentration equivalent to 0.05% choline chloride was used in the following study. Three experiments were conducted to determine whether folic acid functioned in the utilization or in the formation of choline by the chick. The basal diet was fed in these experiments. The methionine content of this diet was found by assay to be 0.49% and the cystine content 0.12%. The addition of 0.1% cystine gave a total methionine-cystine level of 0.71%. The selection of this level of methionine plus cystine was based on the results of West, Carrick, Hauge and Mertz ('51) who found that 0.72 to 0.74% methionine plus cystine gave optimum growth. Thus, the use of 0.71% methionine plus cystine would not supply an excess of methyl groups from methionine, but would permit approximately maximum growth.

The results of the first two experiments are summarized in table 2. Dimethylaminoethanol and monomethylaminoethanol were supplied in molar concentrations equal to 0.05% choline chloride. The betaine was added to the diet at the molar concentration that would just methylate the dimethylaminoethanol (molar ratio 1:1) and the monomethylaminoethanol (molar ratio 1:2) and form the amount of choline chloride equivalent to 0.05% of the diet. In addition, the level of betaine was increased above that required to methylate the monomethylaminoethanol to determine if the extra betaine would give an increase in growth. These two higher levels of betaine (0.146% and 0.194%) were also fed to two lots of chicks to determine the response to betaine alone. A level of 0.2% choline chloride was added to supply the same number of methyl groups as that supplied by the highest

TABLE 2

The effect of folic acid on the formation and utilization of choline from dimethyl- and monomethylaminoethanol and betaine

EXP. NO.	SUPPLEMENT	NO FOLIC ACID				80 μG FOLIC/100 GM			
		No. chicks	Wt. at 4 wks.	Perosis [1]	Mortality	No. chicks	Wt. at 4 wks.	Perosis	Mortality
			gm	%	%		*gm*	%	%
1, 2	None	40	117 ± 3.31 [2]	100	2.5	40	220 ± 5.41	93	2.5
1, 2	0.05% choline chloride	40	194 ± 5.55	77	0	40	295 ± 7.27	5	0
2	0.2% choline chloride	20	272 ± 10.21	50	10	20	321 ± 9.48	0	0
1	0.146% betaine	20	131 ± 5.40	94	10	20	216 ± 11.24	66	10
2	0.94% betaine	20	165 ± 6.63	100	10	20	241 ± 11.24	85	0
1	0.032% dimethyl.[3]	20	174 ± 9.57	88	15	20	269 ± 12.73	25	0
1	0.032% dimethyl. + 0.048% betaine (molar ratio 1: 1)	20	173 ± 7.33	89	10	20	293 ± 8.13	20	5
1, 2	0.027% monomethyl.[4]	40	158 ± 5.56	88	12.5	40	259 ± 6.57	38	0
2	0.027% monomethyl. + 0.097% betaine (molar ratio 1: 2)	20	235 ± 5.57	80	0	20	316 ± 8.01	0	5
1, 2	0.027% monomethyl. + 0.146% betaine (molar ratio 1: 3)	40	240 ± 6.72	76	5	40	303 ± 7.17	5	5
2	0.027% monomethyl. + 0.194% betaine (molar ratio 1: 4)	20	252 ± 9.04	80	5	20	317 ± 7.82	5	0

[1] Per cent perosis based on the surviving chicks.
[2] Mean ± standard error.
[3] Dimethylaminoethanol.
[4] Monomethylaminoethanol.

level of betaine (0.194%). All of the supplements were provided both with and without folic acid at a level of 80 μg per 100 gm of diet.

The results show that folic acid by itself was not effective in the prevention of perosis. A level of 0.05% choline chloride in the absence of folic acid reduced perosis to a small degree. Monomethylaminoethanol plus betaine at all levels was effective to the same degree. Betaine gave some growth response in the absence of folic acid but afforded no protection from perosis.

In the absence of folic acid, dimethylaminoethanol was just as effective as dimethylaminoethanol plus betaine in promoting growth and preventing perosis. The 0.2% level of choline chloride gave a marked growth response. However, the chicks fed the diet containing the monomethylaminoethanol at a concentration equivalent to 0.05% choline chloride plus the higher levels of betaine grew almost as well. All of the chicks receiving monomethylaminoethanol plus betaine in the absence of folic acid showed greater gains than the chicks receiving 0.05% choline chloride or the dimethylaminoethanol plus betaine equivalents to 0.05% choline chloride.

In the presence of folic acid, the dimethylaminoethanol plus betaine and the monomethylaminoethanol plus betaine were just as effective as choline for growth and the prevention of perosis. The monomethylaminoethanol and dimethylaminoethanol, in the presence of folic acid, also gave some growth response and showed some anti-perotic effect. This effect was attributed to the formation of a small amount of choline from these compounds and the dietary methionine or from methyl synthesis. The results show that folic acid is not needed in the transfer of a methyl group from betaine to a precursor of choline. However, folic acid was found to be essential along with choline or its precursors in the prevention of perosis.

In the last experiment, a level of 0.2% choline chloride and an equimolar concentration of its precursors were used. In

addition, two excessively high levels of choline chloride (0.4% and 0.8%) were added to see if, by mass action, the choline would exert an anti-perotic effect in the absence of folic acid.

The results of this experiment are presented in table 3. In the absence of folic acid, betaine and monomethylaminoethanol supplied at levels which were equivalent to 0.2% choline chloride, gave a marked growth response over the

TABLE 3

The effect of folic acid on the formation and utilization of high levels of choline

SUPPLEMENT	NO FOLIC ACID		80 μG FOLIC/100 GM	
	Wt.	Perosis [1]	Wt.	Perosis
	gm	%	*gm*	%
None	113 ± 4.2 [2]	100 (12) [3]	186 ± 7.85	100 (18)
0.2% choline chloride	236 ± 13.70	71 (14)	316 ± 8.34	5 (19)
0.384% betaine	158 ± 7.30	94 (18)	214 ± 7.21	95 (20)
0.108% monomethyl.[4]	118 ± 4.88	43 (14)	265 ± 9.28	25 (20)
0.108% monomethyl. + 0.384% betaine	278 ± 6.14	53 (19)	339 ± 13.37	5 (19)
0.4% choline chloride	254 ± 8.18	58 (19)	303 ± 14.82	0 (20)
0.8% choline chloride	255 ± 11.14	63 (19)	313 ± 6.83	0 (20)

[1] Per cent perosis is based on surviving chicks.

[2] Mean ± standard error.

[3] The numbers in parentheses indicate the survivors out of 20 chicks started.

[4] Monomethylaminoethanol.

0.2% level of choline chloride. Levels of 0.4 and 0.8% choline chloride gave insignificant growth responses over the 0.2% level of choline chloride.

In the absence of folic acid, these higher levels of choline chloride were no more effective than the 0.2% level of choline chloride or equivalent amounts of its precursors in the prevention of perosis. These results show that folic acid, as well as choline, is necessary for the prevention of perosis.

DISCUSSION

Choline *per se* has been shown repeatedly to be required for the prevention of perosis in chicks. The results of these

experiments showed that, in the absence of folic acid, monomethylaminoethanol plus betaine is just as effective as choline in the prevention of perosis and is superior to choline in the promoting of growth. It is evident, therefore, that folic acid is not necessary for the transfer of methyl groups from betaine to monomethylaminoethanol to form choline as measured by the incidence of perosis in the chick.

Stekol, Weiss and Weiss ('52) found that in the rat, folic acid or its physiological derivative was involved in the synthesis of both moieties of choline from serine and, through serine from glycine. However, the inability of the chick to place the first methyl group on the ethanolamine moiety of choline (Jukes, '41; Schaefer, Salmon and Strength, '51) excludes the formation of ethanolamine as the fundamental mechanism by which folic acid spares the choline requirement of the chick. The fact that betaine gave a consistently greater percentage growth response in the absence of folic acid than in its presence, indicated that folic acid may be influencing methyl synthesis in the chick and in the absence of folic acid betaine serves as a source of methyl groups.

The growth response obtained with choline, in the absence of folic acid was always smaller than that obtained with monomethylaminoethanol plus betaine under similar conditions. Dubnoff ('49) and Muntz and Herrwitz ('50) showed that choline is first oxidized to betaine before transfer of a methyl group occurs. Dinning et al. ('50 and '51) and Williams ('51) have shown that folic acid or its physiological derivative is required to maintain the activity of the choline oxidase system. Thus, in folic acid deficiency in a diet borderline in methionine, the chicks may be deficient in labile methyl groups because of reduced choline oxidase activity. This deficiency of methyl groups is overcome when betaine is added.

The results of this investigation indicate that folic acid may be required for the utilization of choline in the prevention of perosis. Excessively high levels of choline were ineffective in preventing perosis in the absence of folic acid

(table 3). Although the mechanism by which choline and folic acid prevent perosis is obscure, the relationship of folic acid to purine synthesis (Welch and Nichol, '52) suggests that a possible role of folic acid may be due to the synthesis of a purine-containing coenzyme. This coenzyme may be part of the system that also requires niacin and manganese for the prevention of perosis.

SUMMARY

Experiments were conducted with chicks to compare the antiperotic property of choline with that of betaine plus the methylaminoethanol compounds in the presence and absence of dietary folic acid.

The choline requirement of the particular strain of RIR × BPR crossbred chicks used as experimental subjects appeared to be satisfied with the addition of 0.05% choline chloride to a diet containing less than 0.007% choline when supplemented with adequate folic acid. When the diet was deficient in folic acid, levels of choline chloride up to 0.8% of the diet did not prevent perosis or promote maximum growth.

In the presence of adequate choline, the chick's requirement for supplementary folic acid was not more than 30 μg per 100 gm of diet. In the absence of choline, the folic acid requirement for growth was increased but was not more than 80 μg per 100 gm of diet for supplementary folic acid. High levels of folic acid did not protect the chicks from perosis in the absence of choline.

The chicks were able to utilize monomethylaminoethanol plus betaine as efficiently as an equimolar concentration of choline, either in the presence or absence of folic acid. The results demonstrated that in the chick folic acid is not concerned with the transfer of methyl groups from betaine to form choline. Folic acid was found, however, to be essential along with dietary choline or choline formed from dietary betaine plus monomethylaminoethanol for the prevention of perosis.

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POTASSIUM DEFICIENCY IN THE RABBIT AS A CAUSE OF MUSCULAR DYSTROPHY [1]

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THREE FIGURES

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A paralysis of the neck and legs of dogs deficient in potassium was noted by Ruegamer, Elvehjem and Hart ('46). The histologic study of this syndrome by Smith, Black-Schaffer and Lasater ('50) showed typical hyaline or "waxy" degeneration of the striated musculature, but no lesions in the nervous system. In rats, similar muscle lesions have been noted in potassium deficiency (Cohen, Schwartz and Wallace, '52). An abstract by Flipse et al. ('48) reported that calves suffering from an experimentally induced paralysis could be cured by potassium as well as by biotin supplements. The muscle of the heart is also affected by potassium deficiency; this myocardial necrosis was first described for the rat, and shown in histologic detail, by Schrader, Prickett and Salmon ('37). In addition to the heart lesions, the latter workers noted distention, atony, lack of peristalsis, and general wateriness of the intestines. The occurrence of this symptom has been confirmed by Perdue and Phillips ('52) as well as others.

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It has not been universally accepted that lesions of the striated muscles occur as a sequel to dietary potassium deprivation. The work of the Johns-Hopkins group on this point has been reviewed by Follis ('48), who concluded that the only lesions attributable to potassium deficiency were myocardial necrosis and necrosis of kidney tubular epithelium.

The loss of muscular function in the potassium deficiencies has been termed paralysis because it is generally known that potassium is intimately involved in nerve impulse transmission and with acetylcholine synthesis in neural tissue (Brink, '53). The available evidence indicates that the potassium-deficiency paralysis and the vitamin E-deficiency muscular dystrophy are indistinguishable in major points of symptomology or muscle and nerve histopathology. The occurrence of a muscular dystrophy in chronic choline-deficient rabbits was described by Hove and Copeland ('54), who revived the hypothesis that any disturbance in acetylcholine formation or function results in muscular dystrophy because of the impaired nerve impulse transmission to the muscle.

A frank potassium deficiency has not been described for the rabbit, although Wooley ('54) and Wooley and Mickelsen ('54) reported suboptimum growth due to potassium insufficiency in rabbits reared on purified diets containing a conventional salt mixture that furnished 0.4% potassium in the diet. The present paper reports the results of a study on the potassium deficiency in rabbits.

EXPERIMENTAL

The composition of the basal potassium-deficient diet, R-43, was as follows: extracted casein, 20; sucrose, 57; non-nutritive fiber (cellulose), 10; lard, 6; cod liver oil, 2; calcium phosphate (monobasic), 3.6; magnesium sulfate, 0.65; sodium chloride, 0.55; iron citrate, 0.15; manganese sulfate, 0.02; trace minerals 0.03%. Dry vitamins were added to furnish, per gram of diet: thiamine, riboflavin and pyridoxine, 5 μg

each; calcium pantothenate, 30 μg; niacin, 40 μg; choline chloride, 2 mg; inositol, 0.2 mg; folacin, 1 μg; vitamin B_{12}, 0.05 μg; biotin, 0.1 μg; 2-methyl, 1-4 naphthoquinone, 2 μg; and DL-alpha-tocopheryl acetate, 0.1 mg. This diet contained 0.009% potassium by analysis.

Various levels of potassium were added to diet R-43, as potassium bicarbonate. The acetate or carbonate are too basic, leading to rapid deterioration of the diet and refusal by the animals. Even with adequate potassium, this diet did not allow maximum growth in the rabbit; the average growth rate was between 15 and 20 gm daily. On an identical diet but with extracted soybean meal at 40% as the source of protein (Diet R-14), in place of casein, the average growth rate was about 35 gm daily. The potassium content of the diet R-14 was 0.89%, by analysis, before the addition of sufficient potassium supplement to raise the level to 1.2%.

The superiority of the soybean meal diet, R-14, over the casein diet, R-43K, is evident from the average growth data shown in table 2. Subsequent work has shown that the difference between these diets disappeared when starch replaced sucrose in the casein diet.

The potassium-deficient diets were fed to weanling rabbits (200 to 600 gm body weight) and to young adult rabbits (2,000 gm body weight). Feed and water were available continuously. The animals were weighed thrice weekly. A 24-hour urine sample was collected three times weekly for creatine and creatinine estimations. Blood was drawn by heart puncture from some of the dystrophic rabbits for determination of sodium and potassium employing the Beckman DU Flame spectrophotometer. The plasma and cells were dry-ashed in silica dishes at 450°C. for 8 hours.

At death, or termination of the experiments, the animals were examined for internal gross pathology, and some tissues were preserved in Zenkers solution for possible subsequent histologic study.

RESULTS

The potassium requirement for growth of rabbits

The growing rabbit required between 0.6 and 0.9% of potassium in the diet for maximum growth on the basal diet used (fig. 1). This is much higher than the value (0.18% of the diet) assigned for the potassium requirement of the rat (Shaw and Phillips, '53), and is somewhat more than the established chicken requirement of 0.4% (Burns, Cravens

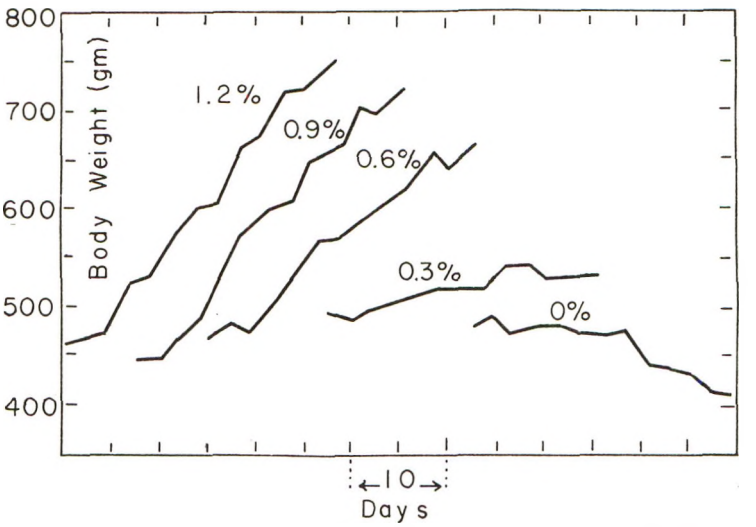

Fig. 1 Effect of graded dietary potassium levels on growth. Each curve is the average of two rabbits fed diet R-43 with the addition to the diet of the indicated level of potassium.

and Phillips, '53). It is of interest to note that no quantitative requirement for ruminants has ever been reported. Such herbivorous animals, normally consuming high potassium foods, may be expected to have a high requirement.

The influence of a high sodium level in the diet on potassium deficiency is shown in table 1. The high sodium diet improved growth very slightly and did not prevent creatinurea and death with the usual pathology.

Adult rabbits placed on the potassium-deficient diet R-43 lost weight rapidly and immediately (table 2). Various ner-

vous disorders were evident before death. During the first week on the basal diet, the rabbits exhibited an abnormal alertness and paced the cage in cat-like fashion. The animals then became quiet, remaining crouched in the corner of their cages, with the appearance of great fright. The ears were

TABLE 1

Influence of excess sodium on the potassium requirement of the rabbit

DIETARY POTASSIUM	DIETARY SODIUM	INITIAL WEIGHT	WEIGHT CHANGE		SURVIVAL TIME		URINARY [1]	
							Creatine	Creatinine
%	%	gm	gm/day		days		mg/kg/day	
0	0.22	310	— 3.4	— 4.6	15	18.0	121	58
		650	— 5.7		21		195	61
0.1	0.22	250	0.5	— 2.6	20	21.5	223	37
		650	— 5.6		23		65	19
0.3	0.22	440	1.7	— 0.5	42	36.5	8	41
		530	2.6		31		237	34
1.2	0.22	300	10.9	18.2	..		5	44
		610	25.5		..		9	43
0	1.02	260	1.9	1.5	21	20.5	204	60
		640	1.0		20		147	70
0.1	1.02	280	2.7	4.2	11	23.5	186	50
		740	5.6		36		..	..
0.3	1.02	270	1.0	4.0	32	42.0	36	60
		650	6.9		52		118	29
1.2	1.02	270	8.2	11.8	..		16	21
		760	14.4		..		9	32

[1] Terminal (within three days of death) values for creatine and creatinine. At 10 days prior to death the average creatine value for all animals was 11.3 mg per kg per day.

TABLE 2

Potassium deficiency in young adult rabbits

POTASSIUM IN DIET	NO. OF ANIMALS	BODY WEIGHT		SURVIVAL TIME	URINARY [1]		TERMINAL PARALYSIS
		Start	End		Creatine	Creatinine	
%		kg	kg	days	mg/kg/day		number
0.009 (R43)	9	2.03	1.22	37	104	28	6
1.20 (R43K)	4	2.10	2.46	(50-killed)	8	39	0
1.20 (R-14)	2	1.98	2.87	(50-killed)	7	40	0

[1] These urinary creatine and creatinine values obtained during the final three days of life. The animals that died without paralysis showed an average creatine excretion of 17 mg daily at this time. None of the rabbits had significant creatine excretion until 10 days prior to death.

laid back, eyes were staring, and the bodies trembled and jerked when touched. After several days of this condition the animals returned to what appeared to be a normal condition, although weight loss continued throughout. Six of the 9 animals developed creatinurea, and a muscular dystrophy that progressed rapidly into complete debilitating paralysis. Three of the rabbits died without developing muscular dystrophy. The average survival time was 37 days.

TABLE 3

Potassium and sodium content of blood and urine

RABBIT STATUS	RED BLOOD CELL		PLASMA		URINE	
	mg/100 ml	*Av.*	*mg/100 ml*	*Av.*	*mg/24 hr./kg*	*Av.*
			1. Potassium content			
Potassium deficient	240		11.0		70	
	224	*214*	17.6	*13.6*	61	*74*
	180		12.3		92	
Control	286		15.0		265	
	278	*282*	19.3	*17.4*	283	*276*
	283		18.0		279	
			2. Sodium content			
Potassium deficient	100		253		37	
	83	*114*	279	*280*	73	*41*
	156		308		11	
Control	76		283		77	
	101	*86*	279	*281*	63	*71*
	82		...		73	

Blood levels of potassium and sodium in deficient rabbits

The sodium and potassium levels of the red blood cells, plasma, and 24-hour urine excretion are given in table 3. As anticipated, the potassium levels were suppressed in the deficient animals. It is of interest to note that the sodium of the red cells increased, apparently replacing part of the lost potassium. A diminished sodium excretion was also noted.

Gross pathology of potassium deficiency in rabbits

Muscular dystrophy. A spectacular paralysis occurred in nearly all rabbits on diets containing 0.3% or less of potassium (fig. 2). This began with unsteadiness of gait and difficulty of regaining normal posture. The "duck waddle" typical of muscular dystrophy was present. Up to this stage the condition was indistinguishable from the muscular dystrophy of vitamin E deficiency. A few animals showed some spontaneous recoveries, but redeveloped the condition in a few days. The dystrophy progressed from the hind quarters to the fore quarters and neck muscles, and in about 6 to 8 days after onset, paralysis was complete and the animal was unable to move. The paralysis was flaccid; every part of the body was limp. This differed from the terminal paralysis of the dystrophy due to vitamin E deficiency, which was spastic and rigid in nature (Hove, '54).

A creatinurea was directly associated with the development of the muscular dystrophy in the potassium-deficient rabbits. The excretion of creatine occurred independently of body weight loss. Young rabbits receiving low levels of potassium developed the dystrophy and creatinurea even though body weight was stationary or increasing slightly (table 1). On the other hand, the three adult rabbits which failed to develop dystrophy, also failed to develop any creatinurea up to the time of death, although as much as 30 to 35% of the original weight was lost (table 2).

Inspection of the leg muscles after death revealed atrophy, a slate-grey color in many cases, and a marked degree of white parallel streaks.

Heart lesions. Nearly all potassium-deficient animals had grossly damaged hearts. In adult animals the heart was flabby and pale. In younger animals areas of myocardial necrosis and extreme scar formation were evident (fig. 3).

Gall bladder. Abnormalities in the gall bladder were prominent. The surface was uneven and had sharp elevations due to small cystoid structures of caseous material in the wall. In all cases the bile contained concretions of numerous

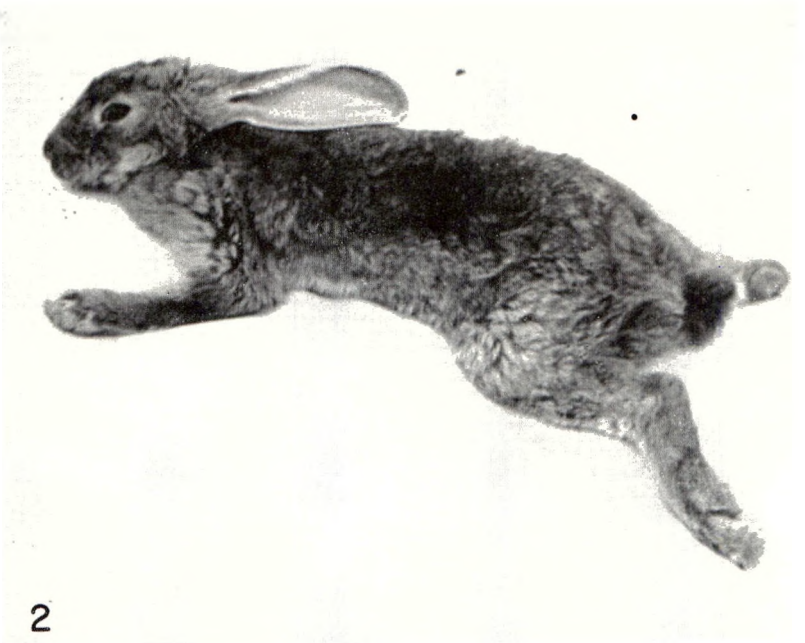

Fig. 2 Potassium deficiency paralysis in the rabbit.
Fig. 3 Myocardial necrosis in rabbit heart as a result of potassium deficiency.

small round whitish-gray balls of soft material, which was partly fatty (cholesterol), partially soluble in dilute acid (calcium), and partially a non-soluble caseous residue. In a few cases, large formless masses of bilirubin-colored concretions were present. At no time were true hard gall stones observed. In a few rabbits jaundice was present, as indicated by distinct yellow color in the subcutaneous tissue and in the atrophied leg muscle.

Stomach lesions. About half of the potassium-deficient rabbits had multiple, small, hemorrhagic lesions in the stomach mucosa.

The intestines. In the potassium-deficient rabbits the intestines were frequently thin, watery, and translucent, with a functionless, unhealthy appearance. Distention of the stomach or intestines was not noted. A similar condition in rats, but much more pronounced, was first described by Schrader, Prickett and Salmon ('37), and was noted also by Henrickson ('51) and others.

No abnormalities were noted, grossly, in the liver, lungs, adrenal glands, sex organs, pancreas, or spleen. The kidneys were frequently swollen and light colored, but without hemorrhage or gross lesions.

DISCUSSION

The most obvious gross symptom of potassium deficiency in the rabbit, aside from the effect on body weight, was muscular dystrophy (or paralysis). It is not without significance that muscular dystrophy and death ensued rapidly even with potassium added to the diet at a level as high as 0.3%; this amount of potassium is more than adequate for the growth of animals such as the rat and pig.

A total of 27 rabbits have died with typical symptoms of potassium deficiency. Of these, 6 were receiving 0.3% dietary potassium, and 4 received 0.1% dietary potassium. No animals receiving these levels of potassium survived. On the other hand death has not occurred in control animals fed the basal diet with 1.2% potassium for as long as 12 months.

Luckily, infectious diseases, parasites, or worms have not yet been observed in any of our animals.

The potassium requirement of the rabbit was more than 0.6% in the diet. This is surprisingly high. To exclude the possibility that this high requirement is needed for the nutrition of bacteria in the cecum of the rabbit, in two young animals the cecum was completely removed surgically. When growth was re-established after the operation, potassium was omitted from the diet. The immediate and drastic weight loss indicated that the cecum played no part in the deficiency. One animal died with the usual symptomology. Recovery in the other rabbit was effected with 0.8% potassium but not with 0.4% potassium. It is of incidental interest to note that the rabbit could grow when the cecum was absent, even though the diet contained 10% fiber. Characteristics of the cecectomized rabbit will be reported in a subsequent publication.

The herbage that normally constitutes the diet of the rabbit is very rich in potassium. This fact may underlie the high requirement, and raises the question as to the potassium requirements of other herbivorous animals of more economic interest.

Muscular dystrophy has been induced in the rabbit by dietary deprivation of three separate nutrients, vitamin E, choline, and potassium. The dystrophy due to choline deficiency was slow to develop and did not proceed to complete disability, as it did in the other two deficiencies (Hove and Copeland, '54).

The three deficiencies resulted in closely similar gross muscle weakness and creatinurea. Onset was most rapid in the potassium deficiency, proceeding to complete paralysis and death in 6 days. Vitamin E deficiency (Hove, '54) resulted in slower development of the dystrophy, which proceeded to immobility and death in about two weeks after onset. Choline deficiency never resulted in complete disability, and produced progressive chronic creatinurea. All three deficiencies resulted in extensive damage to heart muscle. The leg bones of potassium-deficient rabbits were very fragile. Fragility

of the bones also occurred in vitamin E-deficient rabbits (Hove, '54), although the ash content was normal.

Similarity in deficiency symptoms may indicate a common point in the metabolism of vitamin E, potassium, and choline. This point may be acetylcholine production or nerve impulse transmission to striated muscle. Whether smooth muscle is effected in all cases is not clear. However, the atony of intestines and stomach hemorrhages may indicate that potassium deficiency affects innervation of smooth muscle as well as of striated muscle.

SUMMARY

The rabbit required at least 0.6% potassium in the diet for maximum growth. With potassium levels of 0.3% or less, death occurred within 6 weeks with characteristic pathology. High sodium supplements to the potassium-deficient diet allowed slightly better growth but did not prevent death and pathology. Blood-cell sodium content increased while urine sodium decreased in potassium deficiency.

Potassium-deficient rabbits usually developed a severe and rapidly progressing muscular dystrophy, with a closely associated creatinurea. Death occurred after 4 to 6 weeks. Internal pathology, aside from atrophic and streaked musculature of the limbs, consisted of marked myocardial necrosis and scarring, numerous small gall bladder concretions, multiple hemorrhagic areas in the stomach, swollen and pale kidneys, occasional jaundice, and atonic intestinal tract.

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COPROPHAGY IN THE RABBIT

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ONE FIGURE

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Coprophagy is practiced, at least under some conditions, by most rodents; but in the rabbit fecal eating has been stated to be a physiological habit with pecularities that imply a definite relation to the physiology of the digestive system (Frank, Hadeler and Harder, '51). It has been reported that the rabbit excretes a hard and a soft feces with virtually complete consumption of the soft type (Morot, 1882; Madsen, '39; Eden, '40; Southern, '40; Harder, '49); that the soft feces have a higher concentration of the B complex vitamins than does the hard type (Scheunert and Zimmerman, '52; Kulwich, Struglia and Pearson, '53); and that the composition of the soft feces is comparable to that of the cecal contents in protein, crude fiber and other proximate nutrients (Eden, '40; Harder, '49; Frank et al., '51; Olsen and Madsen, '44). None of these reports, however, satisfactorily explains the physiology of coprophagy with respect to its magnitude (Eden, '41), the variability between individuals, the effect of diet, or the effect on digestibility.

In this report data are presented relative to the effect of coprophagy on digestibility and on nitrogen utilization of two diets varying in content of roughage material and chemical composition. The physiology of coprophagy and the com-

positional differences in hard and soft feces are discussed. A method for the determination of the rate of coprophagy and the effect of diet on this rate will be presented elsewhere.

EXPERIMENTAL

A group of 4 Dutch rabbits was divided into pairs, a male and female, with each of the pairs fed one of two diets. The rabbits were placed on their respective diets at 4 weeks of age; at 14 weeks of age they were transferred to metabolism cages and established on approximately a maintenance level of feed intake. The established daily feed allotment was fed in equal portions morning and afternoon. One animal from each dietary treatment was chosen at random and placed in a stanchion. After a preliminary period of 10 days on a constant feed intake and under conditions of confinement, radioactive Cr_2O_3 thoroughly mixed in one day's feed was fed. The fecal excretions were then collected for 10 days from the stanchioned rabbits and for 30 days from the normal animals. The urine and feces for the 4th through the 10th day were composited in all periods for the digestibility and nitrogen balance determinations. At the completion of these collection periods, the situation was reversed for each animal and the procedure repeated. In the course of two years, three groups of 4 rabbits each were studied. The groups were treated similarly except that fecal collections were made mornings and afternoons with group I, mornings with group II, and every two hours for the first 8 days and twice daily thereafter for group III.

The feces were dried for 24 hours at 70°C., air dried for 7 days, weighed, and prepared for radioactive counting by grinding in a Wiley mill to pass a 20 mesh screen.

The basic composition of the two diets was dehydrated grass (roughage diet) and purified components (purified diet). The composition and analyses of these diets are given in table 1.

TABLE 1

Composition of diets

	PURIFIED	ROUGHAGE
	gm per 100 gm	
Crude casein [1]	25.0	...
Dextrin [2]	46.8	...
Cerelose	...	19.0
Dehydrated grass [3]	...	75.0
Hydrogenated vegetable oil [4]	8.0	...
Corn oil [5]	2.0	5.0
Ruffex [6]	10.0	...
Minerals [7]	5.0	...
Trace minerals (Cu, Mn, Fe and I) [8]	0.1	...
Na Cl	...	1.0
Fat soluble vitamins [9]	0.1	...
Water soluble vitamins [10]	3.0	...
Protein [11]	22.4	17.5
Ether extract	10.7	9.1
Ash	4.1	9.9
Lignin	0.49	3.8
Cellulose	10.2	21.0
Other carbohydrates	52.1	38.7

[1] National Casein Sales Co., Chicago, Illinois.

[2] White Dextrin N. F. V., Merck and Co., Inc.

[3] Cerograss, Cerophyl Laboratories, Kansas City, Missouri.

[4] Primex, Proctor and Gamble, Cincinnati, Ohio.

[5] Mazola, Corn Products Refining Co., New York, New York.

[6] Fisher Scientific Co., Pittsburgh, Pennsylvania.

[7] Hawk-Oser salt mixture.

[8] Fe $C_6H_5O_4 \cdot 1\frac{1}{2}\ H_2O$, 453.85; $CuSO_4 \cdot 5H_2O$, 28.15; $MnSO_4 \cdot H_2O$, 16.50; KI, 1.50 gm per kilogram.

[9] Vitamin A palmitate, 666 I.U.; calciferol, 0.02 mg; alpha-tocopherol, 7.5 mg; Menadione, 0.075 mg per 100 gm of diet.

[10] Thiamine, 0.7; riboflavin, 0.7; calcium pantothenate, 1.5; pyridoxine, 0.7; niacin, 20.0; choline, 100.0; betaine, 100.0; inositol, 10.0; p-amino-benzoic acid, 0.2; folic acid, 0.10; biotin, 0.05 mg and vitamin B_{12}, 5 μg per 100 gm of diet.

[11] Analytical data are expressed on a dry matter basis.

RESULTS

The rabbits submitted to the restraint of the stanchion with minimum opposition. Restraint did not influence food consumption of the animals fed the roughage diet, and was decreased in only one rabbit receiving the purified diet. The stanchions effectively prevented the rabbits from eating feces.

A typical pattern of fecal excretion by a rabbit fed the roughage diet is shown in figure 1. The excretion of hard and soft feces is a consistent daily phenomenon both as to time and quantity. Under the conditions of this study, soft feces were eliminated at night and the hard feces during the day.

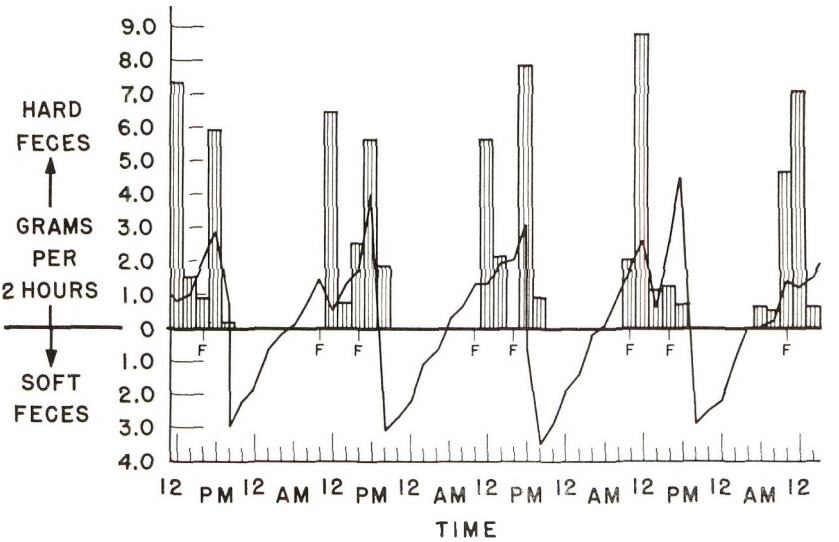

Fig. 1 Feces excreted in two-hour periods by a rabbit on the roughage diet. Bar diagram represents feces collected in free state. Line diagram represents feces collected in stanchioned state. Hard feces are plotted above the base line; soft feces below base line. F denotes time of feeding (10 A.M. and 4 P.M.).

The maximum excretion of soft feces occurred in the initial two hours then gradually decreased in quantity over an interval of 6 to 8 hours, and the elimination of hard feces gradually increased to a maximum. The feces collected in the normal period were entirely of the hard type. In this period the elimination of dry matter had two maxima; one possibly associated with the consumption of soft feces, and the other with feed intake.

The soft feces from the roughage diet were generally excreted as clusters of small, soft, moist, discrete pellets with a distinctive sheen. The hard feces of the same period were

similar to the usually observed hard, round, dry pellets. The purified diet yielded feces that were difficult or impossible to differentiate into hard and soft fractions.

After the rabbits were removed from the stanchions, it was observed that the apparent quantity of fecal excretions had decreased. Quantitative collections indicated that after release from the stanchion the fecal excrement of the rabbits was less than 0.5 gm dry matter on the purified diet for

TABLE 2

Coefficients of apparent digestibility and retention of nitrogen by stanchioned and normal rabbits

	ROUGHAGE DIET			PURIFIED DIET		
	Stanchioned	Normal	Difference	Stanchioned	Normal	Difference
Dry matter	65.5	72.4	6.9 [1] ± 1.1 [2]	83.4	90.4	7.0 [1] ± 1.1 [2]
Protein	49.5	74.4	24.9 [1] ± 1.9	89.6	95.6	6.0 [1] ± 1.27
Ether extract	84.9	86.2	1.3 ± 0.61	97.7	99.1	1.4 [1] ± 0.23
Ash	54.2	61.8	7.4 [1] ± 1.2	51.3	76.0	24.7 [1] ± 4.9
Lignin	3.7	0.20	3.5 ± 5.8	...	...	...
Cellulose	36.4	33.6	2.8 ± 5.1	17.6	41.7	24.1 [1] ± 5.2
Other carbohydrates	93.1	94.2	1.1 ± 1.3	96.9	99.4	2.5 [3] ± 0.88
Nitrogen retained in body (mg day)	18.8	32.3	13.5 [1] ± 2.7	18.8	32.2	13.4 [1] ± 2.7

[1] Highly significant (P ± 0.01).
[2] Standard error of paired difference.
[3] Significant (P ± 0.05).

three to 6 days, and 2 to 6 gm for one to two days on the roughage diet. Whether this was actually a reduced excretion or an increased fecal consumption is not known.

The apparent digestibility of nutrients and retention of nitrogen in the stanchioned and normal periods for both diets is shown in table 2. The difference and the standard error of the difference between these paired variates (normal minus stanchioned) is indicated. The consumption of feces from the roughage diets improved the apparent digestibility of dry

matter, protein, ash, and increased the retention of nitrogen (feed nitrogen minus that of the feces and urine). The digestibility of cellulose, ether extract and other carbohydrates was not influenced by ingestion of feces. On the purified diet coprophagy improved the digestibility of all nutrients studied and increased the retention of nitrogen.

The dry matter excreted on the roughage diet in the form of soft and hard feces in the stanchioned period and in the form of hard feces in the normal period is shown with its composition in table 3. Inspection of these data reveals that similarity of the composition of the hard feces derived from the roughage diet with and without coprophagy, and a considerable difference in the composition of the soft and hard feces from the stanchioned period in protein, lignin, cellulose and other carbohydrates. Similar data are presented for the purified diet except that the figures for the stanchioned period refer to the composition of the combined soft and hard feces. The dry matter eliminated in the stanchioned period contained more protein and other carbohydrates and less lignin and cellulose than did the dry matter excreted in the normal period.

Although the difference in the composition of the hard and soft feces has been observed by others, a reasonable explanation for this difference has not been advanced. Any postulation conceived must be physiologically tenable, and explain the cyclic excretion of hard and soft feces. It has been demonstrated (Eden, '40; Harder, '49; Frank et al., '51) that there is a marked similarity in the composition of the soft feces and the cecal contents. It is reasonable to presume, then, that soft feces originate from the cecum. Anatomically, however, all ingesta from the small intestine must enter the cecum through the ileo-cecal valve. If it is postulated that a segmented contraction of the spiral muscle of the cecum occurs associated with intestinal contents entering the cecum from the ileum, part of this material will be forced in the direction of the sacculated colon and part toward the blind end of the cecum. The ingesta forced into the colon by this

TABLE 3

Average daily composition of fecal dry matter

	ROUGHAGE DIET			PURIFIED DIET	
	Stanchioned		Normal	Stanchioned hard + soft	Normal
	Soft feces	Hard feces			
Dry matter, gm	6.89 ± 0.76 [1]	9.824 ± 0.67 [1]	13.67 ± 1.05 [1]	4.55 ± 0.31 [1]	2.91 ± 0.42 [1]
Protein, %	37.41 ± 3.21	18.73 ± 1.71	16.6 ± 1.38	16.0 ± 0.35	10.3 ± 0.87
Ether extract, %	3.5 ± 0.12	4.3 ± 0.06	4.5 ± 0.16	1.5 ± 0.17	1.0 ± 0.30
Ash, %	13.1 ± 0.34	13.2 ± 0.81	13.9 ± 0.77	10.2 ± 0.63	9.7 ± 1.5
Lignin, %	7.5 ± 0.51	12.3 ± 0.40	12.7 ± 0.76	4.5 ± 0.25	8.7 ± 1.0
Cellulose, %	27.2 ± 7.60	46.6 ± 1.77	45.3 ± 1.70	58.2 ± 2.1	67.7 ± 0.89
Other carbohydrates, %	11.3 ± 1.57	4.9 ± 0.94	7.0 ± 2.16	9.7 ± 1.3	3.2 ± 1.1

Ratio soft: hard (stanchioned).
Lignin content = 0.61 ± 0.045.[1]
Specific activity = 0.51 ± 0.042.[1]

[1] Standard error.

action will form the principal substance of the hard feces with material that has in effect by-passed the cecum. The distribution of radioactivity in the gastrointestinal tract (table 4) supports such a movement of intestinal contents. The ingesta that remain in the cecum, then, form the soft feces when this organ, in a cyclic manner, undergoes a strong contraction of the spiral muscle. This action, perhaps associated with increased activity of the colon, carries the fecal material through the large intestine at a rate that results in the formation of small pellets with a high moisture content.

TABLE 4

Distribution of chromic oxide activity in the gastro-intestinal tract of the rabbit

Roughage diet, using data for stomach as a basis of reference

	HOURS AFTER FEEDING		
	2	4	6
Stomach	1.0	1.0	1.0
Duodenum	..	0.4	..
Jejunum	0.3	0.6	0.3
Ileum	0.9	0.9	0.6
Lower cecum	0.7	0.6	0.6
Upper cecum	0.7	0.7	0.6
Appendix	0.1	0.0	0.5
Sacculated colon	0.6	0.8	0.6
Lower colon	0.1	0.8	1.2
Feces	0.0	0.0	0.2

Observation of intestinal activity in a laparotomy experiment indicated that this postulation of the physiology for the formation of hard and soft feces is essentially accurate.

In accordance with this postulation the differences in composition of the hard and soft feces must arise in the cecum. If it is assumed that a secretion is added to the cecal contents perhaps from the cecal wall, appendix, or both, a possible explanation is then available for the composition differences. If the secretion is largely mucoidal in nature, the protein composition of the soft feces would be increased and the lignin and radioactivity content decreased. The ratios of the lignin concentration in the soft and hard feces and of

the specific activity (table 3) indicate a comparable reduction of these two fractions in the soft over that of the hard feces. Furthermore, since the reduction in ether extract and ash content of the soft feces is small, the postulated secretion must also contain lipid and mineral constituents. The increase in the other carbohydrate fraction of the soft feces over that present in the hard feces is further suggestive evidence for the mucoidal nature of the secretion.

In light of this postulation, the decrease in digestibility of protein and retention of nitrogen (table 2) associated with the prevention of coprophagy can be explained on the basis of a higher metabolic fecal nitrogen excretion. On the other hand, the coefficients obtained in the normal period suggest that this metabolic nitrogen is essentially completely digested when consumed.

The apparent digestibility of the cellulose of the roughage diet was not influenced by coprophagy, but the digestibility of the cellulose of the purified diet was approximately doubled by recirculation. The differences in the time the indigestible portion of the food remains in the body, and in fecal consumption rates may explain this apparent paradox. The cellulose of the purified diet was subject to a possible 9-fold increase in the length of time it was exposed to digestive processes in the normal period as compared to the cellulose of the roughage diet.[1]

The excretion of fecal dry matter and the relative dilution of lignin and increase in protein content of soft feces on the roughage and purified diets suggest that the mucoidal cecal secretion is lower on the purified diet than on the roughage diet. On the roughage diet, 6.9 gm of soft feces were eliminated daily with a dilution factor of approximately 0.5 or a presumed secretion in the cecum of 3.5 gm. On the purified diet, approximately 1.5 gm of soft feces were excreted with a dilution factor of 0.4 or a mucoidal secretion of 0.6 gm. This observation is in agreement with the known increase of metabolic fecal nitrogen with increasing fiber content of diets.

[1] Unpublished data.

DISCUSSION

These observations indicate that coprophagy has a profound effect on the utilization of nitrogen in the rabbit. Whether or not this effect can be directly related to the utilization of dietary nitrogen is a matter of conjecture at the present time. In order for the higher concentration of the nitrogen in the soft feces to be the result of the accumulation of feed residue nitrogen by bacterial action, a reduction in dry matter must be assumed. A reduction in dry matter would result in an increase in the lignin and radioactivity of the soft feces. Since the concentration of the lignin and the activity are reduced in the soft feces only two hypotheses are possible: the addition of nitrogen to the cecal contents or the greater removal of nitrogen in the colon from the hard feces than the soft feces.

While synthesis of bacterial protein occurs in the cecum, the nitrogen for this synthesis is contributed from both the feed residues and the postulated secreted protein. Studies relative to the synthesis of amino acids in the cecum are subject to misinterpretation until the question of the sources of nitrogen in the soft feces is resolved. Kulwich et al. ('54) recently reported that the soft feces of collared rabbits contained a greater proportion of a dose of S^{35} administered by stomach tube than did the hard feces. If it is assumed that the 12 μg dose of sulfate was essentially completely absorbed before reaching the cecum, the S^{35} in the soft feces would then have originated from the cecal secretions.

The possible nutritional benefits the rabbit derives from the apparent physiological habit of coprophagy do not necessarily reside only in the increased utilization of dietary nutrients. The studies of Scheunert and Zimmermann ('52), and Kulwich, Struglia and Pearson ('53), indicate that the vitamin nutrition of the rabbit is enhanced by this habit.

SUMMARY

The rates of coprophagy and the utilization of dietary nutrients were studied in the rabbit on two diets of different

compositions using radioactive Cr_2O_3 as an indicator. A possible physiological mechanism was postulated to explain the production of soft and hard feces, and a protein-containing cecal secretion was suggested to account for the compositional differences in the soft and hard feces.

The prevention of coprophagy resulted in an apparent decrease in protein digestibility and in nitrogen retention, and in the digestibility of dry matter on all diets studied. The effect of this habit on the utilization of other dietary nutrients depended on the diet studied. An increase in the digestibility of the cellulose of a purified type of diet was associated with the longer half-life of feed residues in the digestive tract of rabbits practicing coprophagy in comparison with those in which it was prevented.

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VITAMIN E AND REPRODUCTION IN TURKEYS[1]

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Adamstone ('31) reported a peak of embryonic mortality on the 4th day of incubation in eggs from hens fed a diet low in vitamin E. Embryonic death resulted from the formation of a lethal ring in the blastoderm which destroyed vitelline circulation. Card et al. ('30) fed Rhode Island Red pullets a natural type all-mash diet treated with ferric chloride to destroy vitamin E and reported a high embryonic mortality. It has been reported (Dju et al., '50) that the laying hen requires a minimum of about 1.2 mg/day of D-alpha tocopherol. Singsen et al. ('54) maintained breeding hens on a low-fat, semi-purified vitamin E-deficient diet for a period of 9 months. This ration was also known to be borderline or deficient in several of the B-complex vitamins. Egg production was not influenced by the deficient diets. Fertility was improved by alpha-tocopherol in the absence of the complex vitamin mixture, but not in the presence of the same mixture. Positive encephalomalacia, as diagnosed by histological examination, was produced in a small number of day-old chicks and pipped but unhatched, 21-day embryos. Chicks hatched from these vitamin E-deficient breeding hens, and fed a vitamin E-low diet containing 2% fish oil, consistently

[1] This work was supported in part by grants-in-aid from Distillation Products Industries, Rochester, New York; Philip R. Park, Inc., San Pedro, California and Western Condensing Company, Appleton, Wisconsin.

[2] Public Health Research Fellow of the National Cancer Institute.

showed from 33 to 67% mortality. This mortality was due to encephalomalacia and could be prevented by the addition of high levels of alpha-tocopherol to the chick diet.

Very little work has been done on the nutritional requirements of the breeding turkey. An improvement in hatchability was obtained (Atkinson et al., '51, '53) by the addition of 5% of a liver fraction to an all-vegetable protein ration being fed to turkey hens maintained on wire floors. No improvement in hatchability was obtained (Couch et al., '54) by the addition of fish meal or distillers dried solubles or both to an all-vegetable protein ration fed bronze hens maintained on litter. Hatchability dropped to approximately 10% for all groups after the 12th week. Jensen ('53) in an experiment with Empire White Turkey hens reported an improvement in hatchability from between 30 and 40% up to 80% by the addition of alpha-tocopheryl acetate to a practical type breeder mash. When the alpha-tocopheryl acetate was removed from the diet, the hatchability dropped from about 80 to 45%. This is not surprising in view of reports (Jungherr et al., '49, '52) of the occurrence of field encephalomalacia in chicks fed practical type rations.

The following experiments were designed to study the effect on production, fertility and hatchability of Beltsville Small White turkey eggs of adding alpha-tocopheryl acetate, fish solubles and dried whey to an all-vegetable protein ration supplemented with vitamins and minerals.

EXPERIMENTAL PROCEDURE

Seventy-two Beltsville Small White turkey hens were distributed at random among 8 groups of 9 hens each. These birds had been reared in electrically heated batteries with raised screen floors for 6 weeks, in standard growing batteries with raised wire floors from the 6th through the 12th week, and in pens with raised wire floors for the remainder of a 30-week period prior to the initiation of this experiment.

The diet fed during the first 10 weeks consisted of 55% soybean oil meal, 20% ground yellow corn, 19% ground milo,

3% dicalcium phosphate, 2.5% ground oyster shell and 0.5% salt. This diet was supplemented with the following vitamins as indicated on a per pound basis: 2 mg riboflavin, 12.5 mg calcium pantothenate, 20 mg niacin, 400 mg choline chloride, 6 μg vitamin B_{12}, 2 mg menadione, 160 mg manganese sulfate, 4500 I. U. vitamin A and 1200 I. C. U. vitamin D_3. From the 10th through the 30th week, soybean oil meal was decreased to 40% and ground yellow corn and ground milo were increased to 25 and 29% respectively. The diet was modified at the time the present experiment was initiated to the extent that soybean oil meal was decreased to 25% and ground yellow corn and ground milo increased to 35 and 34% respectively, and the vitamin A was increased to 9000 I. U. per pound. The source of vitamin A used in this experiment was a stabilized product in which the vitamin A had been "sealed in" through the use of hydrogenated vegetable fat.

Each group of 9 birds was placed in a 10′ × 12′ pen with a 1″ × 2″ raised wire floor. Four nests were placed in each pen. Feed and water were supplied ad libitum. Each pen of females was mated with a male of the same breed which had been reared on the range of the Poultry Husbandry Department of the Texas A. and M. College System. Toms were rotated weekly to obtain maximum fertility. Groups 1, 3, 5 and 7 were fed the basal diet unsupplemented, supplemented with 5% condensed fish solubles, with 3% dried whey and with 5% condensed fish solubles and 3% dried whey, respectively. Groups 2, 4, 6 and 8 were fed the same diets as groups 1, 3, 5 and 7 with the additional supplement of D-alpha-tocopheryl acetate [3] at a level of 20 mg/lb. in each group.

Eggs were collected at 4 stated intervals each day, were kept in a constant temperature egg cooler at 55°F. and were set once each week. Eggs were candled at 7, 14, 21 and 24 days and all infertile eggs and those containing dead embryos were removed so that time of embryonic death could be determined. Eggs which were removed during incubation and those which failed to hatch on the 29th day of the incubation

[3] Myvamix, Distillation Products Industries, Rochester, New York.

period were broken out and a gross macroscopic examination was made of the embryos.

The alpha-tocopheryl acetate was removed from the diets of groups 2, 4, 6 and 8 at the end of 9 weeks and was added to groups 1, 3, 5 and 7 at that time. This was done so that supplements other than alpha-tocopheryl acetate could be evaluated over the entire experimental period and so that information on storage of alpha-tocopheryl acetate by the hens could be obtained.

A sample of 4 eggs from each experimental group was sent to Distillation Products Industries for assay of tocopherol content during the 9th week of the experimental period. Additional samples were obtained for analysis 8 weeks after the diets were reversed. The method of analysis for the tocopherol content of the eggs was by the ethanol-petroleum ether extraction procedure described by Dju et al. ('50). The tocopherol content of the yolks and egg whites was determined separately.

RESULTS AND DISCUSSION

It is quite obvious from tables 1 and 2 that the supplements employed, other than alpha-tocopheryl acetate, had no effect on egg production, fertility or hatchability during the entire 17-week period covered by the present study. The addition of 20 mg of alpha-tocopheryl acetate per pound of feed increased hatchability approximately 36% during the first 9 weeks (table 1). When the hatchability data (table 1) were treated statistically by analysis of variance, a significant difference was found between treatments. An F value of 13.82 was obtained for treatments whereas only 3.02 was needed for significance at the 0.01 level of probability. On further analysis an F value of 90.35 was obtained for vitamin E versus no vitamin E whereas only 7.17 was needed for significance at the 0.01 level of probability. This indicates that under the conditions of the above experiment vitamin E was needed and when it was supplied hatchability reached a normal level.

TABLE 1

Effect of D-*alpha-tocopheryl acetate and unidentified factor sources on egg production, fertility, hatchability and tocopherol content of egg yolks of Beltsville small white turkey eggs* [1]

GROUP NO.	SUPPLEMENTS TO BASAL DIET	PRODUCTION	EGGS SET	FERTILITY	HATCH FERTILE EGGS	TOC./EGG YOLK
		%		%	%	μg
1	None	48.3	202	98.5	54.3	195
2	D-alpha-tocopheryl acetate (20 mg/lb.)	34.9	198	98.5	88.2	823
3	Fish solubles (5%)	38.6	187	65.8	47.2	273
4	Fish solubles (5%) + D-alpha-tocopheryl acetate (20 mg/lb.)	37.0	198	96.0	93.2	736
5	Dried whey (3%)	33.7	159	98.7	51.6	156
6	Dried whey (3%) + D-alpha-tocopheryl acetate (20 mg/lb.)	39.7	167	92.8	85.8	922
7	Dried whey (3%) + fish solubles (5%)	44.4	174	99.4	52.0	174
8	Dried whey (3%) + fish solubles (5%) + D-alpha-tocopheryl acetate (20 mg/lb.)	33.7	172	97.7	83.9	870
	Effect of supplement other than D-alpha-tocopheryl acetate					
1,2	None	41.6	400	98.5	71.1	
3,4	Fish solubles (5%)	37.8	385	81.3	75.1	
5,6	Dried whey (3%)	35.4	326	95.7	68.6	
7,8	Fish solubles (5%) + dried whey (3%)	39.1	346	98.6	67.7	
	Effect of D-alpha-tocopheryl acetate					
1,3,5,7	None	41.3	722	90.3	51.7	200
2,4,6,8	D-alpha-tocopheryl acetate (20 mg/lb.)	36.3	735	96.3	88.0	838

[1] Data from first to 9th week, inclusive. Tocopherol content is of eggs collected during the 9th week.

TABLE 2

Effect of D-*alpha-tocopheryl acetate and unidentified factor sources on egg production, fertility, hatchability and tocopherol content of egg yolks of Beltsville small white turkey eggs* [1]

GROUP NO.	SUPPLEMENTS TO BASAL DIET	PRODUCTION	EGGS SET	FERTILITY	HATCH FERTILE EGGS	TOC./EGG YOLK
		%		%	%	μg
1	D-alpha-tocopheryl acetate (20 mg/lb.)	39.5	164	89.6	63.3	590
2	None	31.1	108	99.1	79.4	208
3	Fish solubles (5%) + D-alpha-tocopheryl acetate (20 mg/lb.)	28.2	112	96.4	72.2	777
4	Fish solubles (5%)	24.7	82	98.8	80.2	265
5	Dried whey (3%) + D-alpha-tocopheryl acetate (20 mg/lb.)	28.7	102	37.3	65.8	980
6	Dried whey (3%)	37.7	145	55.9	71.6	187
7	Dried whey (3%) + fish solubles (5%) + D-alpha-tocopheryl acetate (20 mg/lb.)	27.8	89	74.2	75.8	642
8	Dried whey (3%) + fish solubles (5%)	28.7	123	44.7	85.5	200
	Effect of supplement other than D-alpha-tocopheryl acetate					
1,2	None	35.3	272	93.4	70.1	
3,4	Fish solubles (5%)	26.5	194	97.4	75.7	
5,6	Dried whey (3%)	33.3	247	48.2	69.7	
7,8	Fish solubles (5%) + dried whey (3%)	28.3	212	57.1	80.2	
	Effect of D-alpha-tocopheryl acetate					
1,3, 5,7	D-alpha-tocopheryl acetate (20 mg/lb.)	31.1	467	76.9	68.5	747
2,4, 6,8	None	30.6	458	70.7	78.7	215

[1] Data from 10th to 17th week, inclusive. Tocopherol content is of eggs collected during the 17th week.

Hatchability of eggs from hens in groups that were not fed diets supplemented with vitamin E during the first 9 weeks (groups 1, 3, 5 and 7) was increased approximately 17% by supplementation of the diet with alpha-tocopheryl acetate at the beginning of the 10th week of the experimental period (table 2).

The average hatchability of eggs from groups 2, 4, 6 and 8 was 88% (table 1) for the first 9-week period, during which time all birds in these groups received the supplement of 20 mg of alpha-tocopheryl acetate per pound. Removal of the alpha-tocopheryl acetate from the diet at the end of the 9th week caused the hatchability to decrease from 88 to 78.7% (tables 1 and 2). This would indicate that the turkey hen stores tocopherols to a greater extent than was stated in the report by Jensen ('53). He reported that hatchability of eggs from birds fed alpha-tocopheryl acetate was reduced from about 80 to 45% by the removal of this source of vitamin E from the diet for a period of three weeks.

Alpha-tocopheryl acetate apparently had no effect on either egg production or fertility (tables 1 and 2). Egg production was slightly lower where the alpha-tocopheryl acetate supplement was included in the diet, but this is thought to be due to individual variation within the groups. Fertility was considerably lower in group 3 than in the other groups; however, this is not thought to be due to treatment but probably due to preferential mating on the part of the females. No other explanation is offered for this difference in fertility, and certainly other groups not getting vitamin E gave normal fertility.

All embryos found dead during the incubation period were examined in order to determine time of death and to check for any abnormalities which might occur. There were 309 dead embryos out of 652 fertile eggs during the first 9-week period from hens that did not receive alpha-tocopheryl acetate and there were only 83 dead embryos out of 708 fertile eggs from hens that were fed alpha-tocopheryl acetate. Natural peaks of embryonic mortality occurred on the 4th and 28th

days of incubation in eggs from those groups not supplemented with the vitamin. A large number of embryos from the unsupplemented groups died between the 24th and 28th days of incubation. This was not true for those groups supplemented with alpha-tocopheryl acetate. All deficient embryos dying after the 17th day of incubation were found to be smaller than normal as determined by body measurement. Most of the deficient embryos which died during incubation appeared to be blind and were found to have a cloudy lens or a cloudy spot under the cornea; some of the embryos were found to have both of these conditions. A more complete description of the eye condition has been reported by Ferguson et al. (54). Within two weeks after the diets were reversed the above mentioned abnormalities ceased to occur and none were found in any group during the last 6 weeks of the 17-week experimental period. This would also indicate that there was considerable storage of the vitamin by the birds.

It is apparent from table 1 that the addition of alpha-tocopheryl acetate to the diet of turkey hens increased the tocopherol content of egg yolks at the end of the first 9 weeks of the experiment. Reversal of the diets at the beginning of the 10th week resulted in a lowering of the tocopherol content of egg yolk from hens in groups 2, 4, 6 and 8 and an increase in same from groups 1, 3, 5 and 7 at the end of the 17th week (table 2). Such a decrease in the tocopherol content of the egg yolk did not result in as low a percentage hatchability in eggs from groups 2, 4, 6 and 8 as had been observed earlier in eggs from groups 1, 3, 5 and 7, even during the final week of the experiment. No explanation is immediately apparent for the hatchability remaining at a higher level in groups 2, 4, 6 and 8 since the tocopherol content of the eggs had decreased to a point where much lower hatchability might have been expected. The average hatchability of eggs from groups 2, 4, 6 and 8 was 78.7% for the 17th week of the test while it was 51.7% for groups 1, 3, 5 and 7 during the 9th week prior to reversal of the diets. It should be pointed out, however, that the groups not provided supplementary alpha-

tocopheryl acetate during the first 9 weeks of the test had been reared on an all-vegetable protein diet that was shown by the results herein reported to be low in vitamin E activity. It is also possible that unidentified factors, independent of or interacting with alpha-tocopheryl acetate, may be depleted more slowly than vitamin E.

Late in the season a group of 200 Broadbreasted Bronze turkey eggs were obtained from a commercial turkey breeder who was feeding a commercial turkey breeder mash and who had experienced an extreme drop in both fertility and hatchability. These eggs were incubated in our laboratory. Only 30% of the 200 eggs were fertile and only 41.7% of the fertile eggs hatched. Eight of the embryos which died between the 24th and the 28th day of incubation were found to have a cloudy lens, were smaller in size, and were identical with embryos from dams that did not receive the supplemental alpha-tocopheryl acetate in the present report.

SUMMARY

The addition of alpha-tocopheryl acetate to an all-vegetable protein diet at a level of 20 mg/lb. of feed increased hatchability of Beltsville Small White turkey eggs from 51.7 to 88%. Dried whey and fish solubles had no effect on hatchability over a 17-week period under the conditions of these experiments.

Alpha-tocopheryl acetate had no effect on egg production or fertility under the conditions of the above experiment.

Removal of alpha-tocopheryl acetate from the diet at the end of the first 9 weeks produced a decrease of 9.3% in the hatchability during the remaining 8 weeks of the experimental period. This indicates that vitamin E is stored to an appreciable extent by the mature laying turkey, or possibly that other factors are involved.

The peak of embryonic mortality in eggs from hens where the diets were not supplemented with alpha-tocopheryl acetate was from the 24th to the 28th days of the incubation period. These embryos appeared blind and were found to have a

cloudy lens or a cloudy spot under the cornea. Some embryos had both of these defects. Most deficient embryos which died during the incubation period were found to be smaller in size than the normal.

A much higher tocopheryl content of egg yolks was found in all groups receiving dietary supplements of alpha-tocopheryl acetate (20 mg/lb.) than was found in egg yolks from hens not fed this supplement. No tocopherol was found in the egg whites.

The occurrence of a possible vitamin E-deficieny in the field is reported.

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EFFECT OF LONG TIME FEEDING OF WHOLE MILK DIETS TO WHITE RATS[1]

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In an earlier study rats were maintained for the whole of life upon a diet consisting only of pasteurized cow's milk supplemented with traces of iodine, manganese, copper, iron and a small amount of cod liver oil. Such rats had the same mean span of life as controls maintained upon complex stock diets. (McCay et al., '52). However, at the time of death the bones of the rats fed the milk diet were denser, the soft tissues of the kidneys were somewhat more calcified and the teeth showed no signs of decay.

A series of papers from Wisconsin (Anderson et al., '47) has indicated that milk may have some effect in the prevention of decay of the teeth of cotton rats.

Since the maintenance of strong bones and sound teeth during the later periods of life is of interest, the effects of feeding whole milk throughout life were extended in a new series of experiments reported in this study. Since many infant foods as well as condensed milk contain sucrose dissolved in milk, one of the variables consisted of such a combination.

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[2] U. S. Department of the Interior, Cortland, N. Y.

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[4] Snodgras Laboratory of Pathology, St. Louis, Mo.

PROCEDURE

All milk used was fresh, pasteurized and supplemented with trace elements and cod liver oil as described previously (McCay et al., '52). The following diets were fed to groups of albino rats bred in our own colony and distributed in relation to sex and litter of origin:

1. Milk.
2. Milk containing 10% of sucrose dissolved in it.
3. Milk with the animals having free acess to dry sucrose.
4. Milk and a 10% solution of sucrose.
5. Same as no. 6 but supplemented with 10% by weight of cooked, dried whole eggs.
6. Mixed stock diet of open formula dog feed.[5]

The rats were allowed to consume as much of each diet as they desired. In the case of diet 4, separate tubes of fresh milk and sugar solution, as well as those with fresh milk were provided twice daily.

Sixty rats were fed each diet and the animals were under observation for the whole of the life span. Each of these groups of 60 was subdivided into three groups of 20 rats each, namely males, virgin females and bred females.

The criteria of the effect of the diets were the following:

1. Growth and life span.
2. Consumption of food and liquids.
3. Reproduction and composition of the young.
4. Pathology.
5. X-ray photographs of soft tissues.
6. Decay of teeth at the time of death.
7. Bone density and calcium content per unit volume.

When this study was half completed and the rats were a year old, reports came from our pathologist that rats confined to liquid milk in a preceding study had suffered from

[5] The open formula dog food had the following percentage composition: alfalfa meal 1.5, meat scrap 18, fish meal 1.5, soybean meal 8.5, tomato pomace 2.0, corn flakes 20.3, wheat flakes 5.0, tallow 5, baked wheat flour 20, wheat germ 8.5, milk 4.0, cheese meal 1.5, salts 0.20, dicalcium phosphate 0.75, brewer's yeast 2.0, irradiated yeast 0.10, cod liver oil 0.75, liver meal 1.0, Chlorophyllin 0.075.

hair balls formed in the stomach. Since Howell et al. ('48) had found that cotton rats suffered from such hair balls even when fed solid diets low in roughage and since this condition was alleviated by the feeding of ground cellophane, half of the animals in these studies were given access to ground cellophane in a separate container after they were a year of age. However, this failed to alleviate the condition.

RESULTS

Growth and life span. All animals were weighed at weekly intervals for the first 8 weeks and thereafter at intervals of two weeks until they died in old age. Weight data (table 1) are presented for a few periods of the life span. The mean span of life in days and the mean maximum body weights are summarized for each group in table 2.

The data of table 2 were also tested for significance using the methods described by Duncan ('53). This study was made under the direction of Prof. C. R. Henderson.[6]

Starting at 100 days of age and continuing until the end of life, the rats fed milk containing dissolved sucrose were significantly heavier than those in other groups. The mean body weight usually declines in old age as the chronic diseases such as bronchiectasis increase in severity but this group maintained an increased body weight to the end of life.

The animals of all three subdivisions of group 2, fed sucrose dissolved in milk, had maximum body weights that were significantly greater than those of comparable animals fed any of the other diets.

The maximum body weights attained by animals fed supplements of sucrose in any form were significantly greater than those fed no sucrose. Among the males, the group fed the stock diet supplemented with egg had a maximum body weight that was significantly lower than that of the males fed any other diet.

When Duncan's multiple range test was applied to life span data, it was found that the rats fed diets 4 and 5 lived sig-

[6] Professor of Animal Husbandry, Cornell University.

TABLE 1

Mean body weights of rats in grams at different ages (days) plus standard deviations

DIET NO.	VIRGIN FEMALES				BRED FEMALES			MALES			
	100 days	300 days	500 days	600 days	100 days	500 days	600 days	100 days	300 days	500 days	600 days
1 — Milk	200 ± 3.8	240 ± 4.6	246 ± 6.8	251 ± 9.0	200 ± 1.7	267 ± 6.3	259 ± 8.3	290 ± 4.4	370 ± 6.8	372 ± 9.6	365 ± 17.8
2 — Milk containing sucrose	222 ± 3.9	354 ± 8.0	404 ± 12.3	418 ± 11.3	225 ± 4.7	353 ± 9.1	379 ± 7.8	341 ± 4.2	520 ± 8.6	498 ± 36.3	542 ± 12.4
3 — Milk and dry sugar	194 ± 3.7	274 ± 8.2	318 ± 12.0	329 ± 14.9	194 ± 2.7	305 ± 9.4	319 ± 10.3	280 ± 6.9	428 ± 10.5	447 ± 19.3	398 ± 25.9
4 — Milk and sugar sol.	208 ± 3.2	270 ± 5.5	303 ± 6.4	310 ± 9.3	204 ± 4.5	286 ± 3.3	304 ± 15.2	307 ± 4.9	414 ± 7.3	424 ± 15.3	416 ± 15.1
5 — Stock + egg	211 ± 3.5	250 ± 4.3	257 ± 6.1	267 ± 9.7	213 ± 2.6	272 ± 4.7	277 ± 8.2	266 ± 4.1	330 ± 6.6	340 ± 9.6	365 ± 9.5
6 — Stock	200 ± 3.1	250 ± 5.4	260 ± 9.6	268 ± 10.8	205 ± 3.4	248 ± 7.0	264 ± 6.8	275 ± 2.2	370 ± 7.1	368 ± 13.2	365 ± 11.6

Using Duncan's multiple range test at a 5% level:

	100 days of age	300 days of age — virgins and males only
Standard error of diet mean	3.12	2.46
Standard error of sex mean	2.21	1.40
Standard error of subgroup mean —	5.40	3.48

Significant results:

Diets	2 and 4 greater than all others	2 greater than all others 3 and 4 greater than 1, 5, 6 1 and 6 greater than 5
Sex	Males greater than females	Males greater than virgins

Individual groups:

Virgins —	2 greater than 6, 1, 3; 5 greater than 3
Bred females —	2 greater than 6, 4, 1, 3; 5 greater than 3
Males —	2 greater than all others; 4 greater than 1, 3, 6, 5; 1 greater than 6, 5.

2 greater than all others; 3 and 4 greater than 1, 6, 5
2 greater than all others; 3 greater than 4, 1, 6, 5; 4 greater than 1, 6, 5; 1 and 6 greater than 5.

nificantly longer (5% level) than those fed sucrose dissolved in milk. No significant differences were found between virgin females and those that produced litters.

TABLE 2

Mean life span (days) and mean maximum body weights attained (grams) including standard deviations of all means

DIET NO.	MEAN LIFE SPAN			MEAN MAXIMUM BODY WEIGHT		
	Virgins	Bred females	Males	Virgins	Bred females	Males
1	723±49.1	619±52.1	563±33.8	264± 5.7	266± 8.5	397± 5.6
2	682±31.0	665±30.2	520±17.3	428± 8.6	373±12.2	576± 8.2
3	692±33.5	682±38.7	641±26.9	335±11.7	318±12.3	468±11.8
4	780±25.2	698±34.9	667±31.2	331±10.3	310±11.1	446± 9.5
5	727±23.4	662±38.8	720±34.9	275± 6.6	283± 6.5	367± 7.5
6	698±35.3	717±32.0	638±30.5	276± 7.9	268± 7.8	399± 5.5

Using Duncan's multiple range test at a 5% level:

	Life span	Maximum weight
Standard error of diet mean	— 19.75	5.2
Standard error of sex mean	— 13.97	3.7
Standard error of subgroup mean	— 34.21	9.1

Significant results:

Diets	4 greater than 1 and 2 5 greater than 2	2 greater than all others. 3 and 4 greater than 6, 1, 5.
Sex	all differences significant	all differences significant.

Individual groups:

Virgins	— none	2 greater than all others. 3 and 4 greater than 6, 5, 1.
Bred females	— none	Same as for virgins.
Males	— 5 greater than 1 and 2; 3 and 4 greater than 2	2 greater than all others. 3 and 4 greater than 6, 1, 5. 6 and 1 greater than 5.

The males fed sucrose dissolved in milk had life spans significantly shorter than those of groups 3, 4 and 5. Those males fed the diet supplemented with egg had a significantly longer span of life than those fed either milk alone or sucrose dissolved in milk. The trend of the data for the mean span of life for the overweight females fed sucrose dissolved in

milk was toward a shortening of the length of life but this was not significant when tested by Duncan's methods.

All life span studies require years of tedious repetition but these data confirm an earlier result that rats fed whole milk diets with trace supplements have essentially the same span of life as rats fed complicated diets of mixed foodstuffs. In the second place evidence from this study indicates that male rats fed sucrose dissolved in milk tend to become overweight very early in life and to have significantly shorter spans of life. In the third place these data confirm many earlier observations that the female outlives the male. Finally, the tendency of males fed the stock diet supplemented with eggs, toward a longer life span, has sufficient significance so that we are repeating the experiment with new groups of rats.

In studies of the effects of diet upon life span, the number of individuals that survive and live for a period in old age has some significance. Data indicating the life span for each rat exceeding 800 days of age are given in table 3. Past experience has indicated that there is a hereditary as well as a dietary factor involved. The present data indicate the short lives of males fed sucrose dissolved in milk and the greater survival of males fed the stock diet supplemented with egg. Likewise, in spite of the known high incidence of hair balls in the stomachs of rats fed milk, the survival was just as good as for those fed the stock diet. These latter rats had no hair balls.

Furthermore, there is no evidence for any shortening of the span of life from the long continued consumption of a high cholesterol diet fed in the form of eggs.

Bone size and density. Since the volume of any typical bone has long been known to be correlated with the size of the body of the rat a humerus was carefully dissected from each rat. After freeing from fat by extraction with ether, these bones were coated with a thin layer of zein before determining the volume, density and calcium content. The density and volume of each bone was calculated after weighing the bone in air and suspended in water.

TABLE 3

Life span (days) of old rats that exceeded an age of 800 days

DIET NUMBERS																	
1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Virgins						Bred females						Males					
811	816	828	817	808	848	811	885	810	829	801	801	830	(none	887	804	824	800
813	818	844	822	819	899	844	890	813	835	831	811		reached		806	842	940
888	819	846	830	934	954	878		818	856	852	829		even		840	843	
903	924	846	856	955	968	910		838	861		846		700		872	935	
949		890	861			915		847	954		888		days)			989	
949			872					869	956		896					999	
960			927					891			907						
998			971								936						
1033			991														

The data for mean values are summarized by sexes in table 4. These confirm earlier findings that the female has denser bones when it dies in old age than does the male in spite of the longer life of the female. This difference between the sexes is also reflected in the calcium content per unit volume of bone. This is higher for the female.

Furthermore, these data confirm earlier observations that diets composed mostly of fresh milk provide the animals with denser bones in old age than do the stock diets. This happens in spite of the much higher level of calcium calculated in relation to a dry basis for the stock diet.

The volume of the bones indicates that the animals fed diet 2, milk containing dissolved sucrose, were slightly larger animals although this is only marked in the case of the males. Examination during dissection also indicated that animals fed diet 2 were much fatter than any others, considered as a group.

The consumption of milk and sugar. From previous experiments it was known that the female rat after it has attained adult size consistently consumes more calories and fluid, per unit of body weight than does the male. This was observed previously in the case of rats allowed 4 different forms of fluid, namely, coffee, water, fresh milk and 10% sucrose solution (McCay et al., '52).

The data of table 5 indicate that this trend toward high fluid consumption by the female was already evident at 50 days of age except for one group. After 100 days of age, this difference between the sexes is consistent for the rest of life.

The consumption of sugar by the female is consistently higher than it is by the male. This is evident from experience with diet 3 in which the rats had access to solid sugar and liquid milk. In this group about the same volume of milk was drunk each day and the females consumed their additional food in the form of sucrose. The evidence is less clear in the case of sugar consumed in solution, diets 2 and 4, due to the tendency of the females to ingest more fluid both in the form of milk and 10% sucrose solution.

TABLE 4

Bone density, volume and calcium per unit volume of the humerus including standard deviations of all means

DIET NO.	VIRGINS					BRED FEMALES					MALES				
	Density			Mean volume	Mean Ca	Density			Mean volume	Mean Ca	Density			Mean volume	Mean Ca
	Min.	Max.	Mean			Min.	Max.	Mean			Min.	Max.	Mean		
				ml	*mg/ml*				*ml*	*mg/ml*				*ml*	*mg/ml*
1	1.27	1.44	1.37 ± 0.02	0.2041 ± 0.007	337 ± 12.9	1.27	1.48	1.35 ± 0.02	0.2143 ± 0.005	329 ± 9.4	1.24	1.46	1.32 ± 0.02	0.2834 ± 0.006	321 ± 7.7
2	1.42	1.54	1.50 ± 0.02	0.2151 ± 0.005	371 ± 9.0	1.37	1.64	1.45 ± 0.02	0.2264 ± 0.004	360 ± 6.9	1.36	1.53	1.42 ± 0.02	0.3261 ± 0.007	344 ± 6.1
3	1.38	1.56	1.46 ± 0.01	0.2098 ± 0.006	356 ± 10.3	1.34	1.45	1.40 ± 0.02	0.2117 ± 0.004	343 ± 11.7	1.27	1.48	1.38 ± 0.02	0.3008 ± 0.009	332 ± 8.2
4	1.45	1.57	1.49 ± 0.02	0.2127 ± 0.007	380 ± 13.9	1.27	1.53	1.37 ± 0.03	0.2309 ± 0.010	344 ± 12.0	1.24	1.51	1.38 ± 0.03	0.2987 ± 0.007	338 ± 9.2
5	1.09	1.36	1.28 ± 0.02	0.2072 ± 0.005	312 ± 5.8	1.18	1.37	1.28 ± 0.01	0.2201 ± 0.003	313 ± 10.6	1.08	1.35	1.19 ± 0.03	0.2794 ± 0.007	280 ± 8.6
6	1.27	1.41	1.33 ± 0.02	0.2041 ± 0.005	332 ± 14.2	1.14	1.36	1.22 ± 0.05	0.2161 ± 0.010	286 ± 27.3	1.11	1.24	1.18 ± 0.02	0.2819 ± 0.004	275 ± 11.5

These data indicate that the taste for sugar does not decline with age. This confirms earlier observations that rats retain their taste for sweet products until they die of old age (McCay and Eaton, '47).

Finally the calories ingested per unit of body weight is constant within each sex as age progresses. Since it is well known that rats decrease their activity as they age and since the table of weight data indicates only modest or no increase

TABLE 5

Consumption of milk (ml) and dry sugar (gm) per day per 100 gm of body weight in relation to age

AGE	DIET	1		2		3		4	
		♀	♂	♀	♂	♀	♂	♀	♂
days									
50	Milk	59	59	43	36	35	32	48	45
	Sugar			4.3	3.6	4.5	3.9	2.5	2.5
100	Milk	42	34	29	26	21	23	32	30
	Sugar			2.9	2.6	3.4	2.5	1.5	1.1
200	Milk	37	27	22	19	17	17	25	14
	Sugar			2.2	1.9	2.3	1.7	2.4	2.5
300	Milk	34	27	21	17	17	16	18	13
	Sugar			2.1	1.7	2.4	1.9	2.7	2.2
400	Milk	34	24	19	15	15	13	16	12
	Sugar			1.9	1.5	2.3	1.6	2.6	2.3
500	Milk	33	27	19	15	16	15	15	12
	Sugar			1.9	1.5	2.0	1.5	2.5	2.1
600	Milk	33		21	19	16	15	17	15
	Sugar			2.1	1.9	2.0	1.6	1.9	2.2

of body weight after middle age, these data afford no indication that the basal metabolism of rats declines in old age in a manner comparable to that for man. Hence, man still seems a unique species in this decline of basal metabolism with age.

The condition of the teeth in old age. The heads of all rats were preserved in formaldehyde and shipped to one of us (C.A.H.S.) for dental examination. The findings are summarized in table 6. No caries were found in the case of the animals fed diets 1 and 2 while a substantial amount was

found in the rats fed sugar separately in diets 3 and 4. This would seem to confirm the Wisconsin observations upon cotton rats, namely that sucrose fed dissolved in milk causes less injury to the teeth than when fed separately. It appears that feeding sucrose in solution is not the chief factor in caries prevention, inasmuch as the rats that were given diet 4 received only a liquid diet consisting of milk in one bottle and a 10% sucrose solution in the other, and developed caries of moderate severity. Hence the ingestion of a water solution of sucrose did encourage some decay. On the other hand, it was found that caries was more severe in the rats of group 3, in which milk and dry sucrose were fed.

TABLE 6

Percentage of rats with decayed molars and the relative severity of the caries

NO.	DIET	MALES	FEMALES	SEVERITY
		%	%	
1	Milk	0	0	None
2	Milk + dissolved sugar	0	0	None
3	Milk + dry sugar	57	60	Very severe
4	Milk + sugar solution	26	36	Moderate
5	Stock + egg	10	7	Slight
6	Stock	17	12	Slight

Since caries in animals fed the stock diets was very mild this evidence indicates that sucrose was involved and that milk has protective action when the sucrose is dissolved in the milk but not if the sucrose is drunk in a separate solution.

In life time studies with rats the question has been raised if the condition of the teeth may influence the consumption of food and hence the length of life. This would seem possible but the current data afford no evidence of relationship between mean span of life and amount of tooth decay. A study of the data for the rats of diet 3 given solid sugar was made since this group exhibited the largest number of animals with severe decay. The mean span of life for females of this entire group was 687 days while this span for those with severe decay of all molars was 688 days. Likewise the

values were 641 days for all males of this group and 673 days for those with severe decay of all molars.

Production of young and the composition of their bodies at weaning. Twenty females fed each of the 6 diets were bred first when 4 months old and again when 9 months old. An attempt was made to breed them again at 13 months of age with failure in all groups.

TABLE 7

Litters born and weaned from each group of 20 females

DIET NO.	BORN	WEANED	FIFTEENTH DAY	
			Mean number alive in litter	Mean wt. of litter
				gm
	First breeding			
1	18	10	4.9	85.8
2	17	13	5.7	115
3	17	12	5.8	126
4	19	16	5.6	126
5	19	18	5.9	149
6	20	19	6.3	156
	Second breeding			
1	18	15	5.9	144
2	2	1	6	121
3	12	5	6	159
4	17	12	5.3	128
5	15	12	6.3	166
6	13	10	6.1	139

In the first breeding the percentage of litters weaned was substantial for all groups but those fed the stock diets were superior indicating that it may have been difficult to ingest enough of the milk diets for satisfactory lactation by all females (table 7).

In the second breeding marked failure in conception was noted in group 2. This is the same group that was the fattest from early in life. These lived upon milk containing dissolved sugar.

At 21 days of age one typical rat of each sex was selected from each litter for chemical analysis to determine if the diets of the mothers during gestation and lactation had affected the dry matter, calcium or phosphorus of the young at weaning.

From the mean weights of the young selected for analysis it was observed that those fed the solid diets had a higher body weight than others and that those fed the milk diets were the lightest.

Typical animals of both sexes were analyzed from 99 first litters and 38 second litters. No differences were found between the calcium and phosphorus content of the opposite sexes at the time of weaning. The mean values of young from mothers fed the milk diets varied from 188 to 219 mg of calcium per rat and from 143 to 176 mg of phosphorus per rat. For those fed the two diets of solid mixed foods the comparable values were 262 to 267 for calcium and 190 to 204 for phosphorus. No differences were found in relation to diet except that the young were larger at the time of weaning when the mothers had been fed the solid food of diets 5 and 6. Hence, the body had stored more calcium and phosphorus.

Gross pathology. The gross pathology did not differ essentially from that described previously for another similar experiment (Saxton et al., '53).

The high incidence of hair balls in the stomachs of rats fed the milk diets was noted previously. This amounted to 20% of the individuals fed the 4 milk diets with none in the groups fed the solid stock diets. Free access to ground cellophane by half of the rats did not correct this condition.

Among the females there was a substantial incidence of cysts of the reproductive organs. These were quite evenly distributed among the 6 groups without any relation to diet. However, this incidence amounted to 13% for the virgin rats and 27% for those that had been bred.

Other abscesses and pathological conditions noted on gross examination are recorded in table 8. These were generally

the same as described in former experiments (Saxton et al., '53). Here one notes the usual high incidence in old albino rats of diseases of the middle ear and lungs. From previous observations it was known that milk diets lead to a greater excretion of calcium through the kidneys and increased calcification of the kidney tissues. In the present table the absence

TABLE 8

Gross pathology — Number of cases

CONDITION	SEX	DIET NO.					
		1	2	3	4	5	6
Ear infections	Virgin	15	13	8	11	10	9
	Bred fem.	15	17	12	9	12	9
	Male	16	15	12	5	17	11
Bronchiectasis	Virgin	12	7	9	5	8	4
	Bred fem.	11	14	6	11	7	4
	Male	15	9	6	7	14	8
Tumor	Virgin	2	1	1		1	4
	Bred fem.	2	2	2	1	3	1
	Male	15	9	6	7	14	8
Distended bladder	Virgin						
	Bred fem.			1 pus	1		
	Male	4	5	3	4		2
Bladder or kidney stone	Virgin	1			1		
	Bred fem.		5	2 kidney abscess			
	Male	4		1			
Periarteritis	Virgin		6	5	1	4	1
	Bred fem.		6	3 slight	4	6	1
	Male		4	4		6	2
Pituitary tumors	Virgin	3	3	4	4	2	3
	Bred fem.		1	3	2		2
	Male	3	1	1	6	3	2
Abscesses	Virgin	5	3	3	5	1	3
	Bred fem.	5	4	6	4	6	6
	Male	3	3	1	3	3	
Miscellaneous tumors	Virgin		3	2	1	5	
	Bred fem.	2	2	1	2	5	2
	Male		2	5	2		3

of any urinary tract stones among animals fed the stock diets is evident while there was a low incidence among the milk-fed rats.

The conditions listed as "periarteritis" were observed mostly in animals that had attained a substantial old age of 750 days or more. This consisted of aneurysmal swellings of the arteries of the mesentery. In some cases there were very large nodules. None were observed in animals that had only milk to drink, namely, in group 1.

SUMMARY

Albino rats were divided into 6 groups of 60 each at the time of weaning. Each of these groups consisted of 20 males and 40 females. Half of the females were bred and half were not. The diets for the first 4 groups consisted chiefly of whole fresh milk supplemented with traces of manganese, iron, iodine, copper and cod liver oil. Group 1 was fed only this milk, group 2 was fed similar milk containing 10% of sucrose dissolved in it, group 3 received the milk and had free access to dry sucrose, group 4 had similar access to a 10% solution of sucrose in water. Group 5 was fed a mixed stock diet supplemented with 10% of its weight of whole, cooked dried-egg, while group 6 was fed only the stock diet. The animals were fed these diets until they died in old age.

From early in life until the end, the rats fed milk containing dissolved sucrose were overweight. Males of this group failed to attain a normal span of life and females failed in reproduction. Animals fed milk diets had denser bones in old age. Those fed milk or milk containing dissolved sucrose had no decayed teeth at the time of death while those drinking milk with free access to either a water solution of sucrose or dry sugar had a substantial incidence of decay. Those consuming the dried sucrose suffered the most from decayed molars. Thus, milk consumed directly with sucrose seems to protect teeth against decay.

No evidence was found of a shortening of the span of life as the result of consumption of 10% of whole egg in the diet and the life span of males seemed substantially increased.

In all groups comparable animals consumed about equal amounts of sucrose. This taste for sucrose persisted until the end of the life span indicating no decline of interest in the sweetness of sugar. Females consumed more calories and more fluids per unit of body weight than males. As age progresses the calorie consumption is relatively constant affording no evidence for a decline in basal metabolism with age in the case of rats.

About 20% of the rats, in groups fed milk diets, had hair balls in the stomach and the feeding of ground cellulose did not modify this condition. Females that had been bred had a higher incidence of abscesses of the reproductive organs than virgin rats. All rats at the end of life, irrespective of the cause of death, suffered from severe involvement of the lungs and ears but no special pathology could be related to the diets.

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THE ROLE OF GLYCINE IN CHICK NUTRITION[1]

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In view of the findings of Anderson et al. ('51) and of Fisher et al. ('54b) on the involvement of niacin in amino acid metabolism, we were interested in studying this relationship further by means of a glycine-induced amino acid imbalance.

Glycine toxicity and growth depression in rats have been reported by Jackson et al. ('28), Sullivan et al. ('32) and Hier et al. ('44), and in the chicken by Patton ('39). No explanation, other than a possible amino acid imbalance, was offered by these authors at that time.

More recently there have been numerous reports demonstrating glycine toxicity or growth depression in both rats and chickens which could be overcome with supplementary niacin (Groschke et al., '48; Groschke and Briggs, '46; Henderson et al., '47; Anderson et al., '51; Anderson and Combs, '52).

Recent progress in 1- and 2-carbon metabolism has also linked the vitamins folic acid and B_{12} with glycine metabolism (Fruton and Simmonds, '53). The problem of glycine toxicity has thus become more complicated since several workers claimed to have alleviated or prevented glycine toxicity on niacin-adequate rations by the administration of folic acid

[1] This report is taken in part from a thesis presented by the senior author to the Graduate School of the University of Illinois in partial fulfillment of the requirements of the Ph.D. degree in Animal Nutrition.

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or vitamin B_{12} (Dinning et al., '49; Stern and McGinnis, '51; Naber et al., '52; Machlin et al., '52; Hsu and Combs, '52).

Unlike the rat which has no dietary glycine reqirement, glycine is an essential amino acid for the chick (Almquist and Mecchi, '40). In a study of the glycine requirement of the chick, Almquist and Mecchi ('42) concluded that 1 to 1.5% glycine were required on a 25% casein diet. These authors considered 2% to be slightly growth depressing. On the other hand, Hegsted et al. ('41) showed that 2% glycine promoted better growth than did 1% (with no growth depression), promoted an increased level of muscle creatine and greatly improved the feathering of a fast-feathering strain of chicks. Glista ('51), in his nitrogen balance study of amino acid requirements for the chick, concluded that only 0.18% glycine was required for positive nitrogen balance but that more was necessary for optimum feathering. He concluded that good feathering is not necessarily correlated with optimum growth.

With the above information in mind, the following experiments were conducted which were to throw new light on the role and requirement of glycine in chick nutrition.

EFFECT OF HIGH GLYCINE LEVELS IN VITAMIN B_{12}-FREE NIACIN-LOW RATIONS ON THE GROWTH OF CHICKS

Experiment 1 was designed to produce a glycine growth depression and to study the effect of niacin or vitamin B_{12} or both in overcoming this effect. A factorial design was employed with all 8 possible combinations of the three variables: glycine, niacin and vitamin B_{12}.

Male chicks of a NH ♂ × Columbian ♀ mating were maintained in group batteries. For the first three days they had been fed a low tryptophan-niacin hydrolyzed casein diet. From the third day on they were reared in the group batteries and fed the basal diet employed in the present experiment. On the 7th day the birds were grouped into lots of 10 each according to their weights. They were then placed on their respective diets, accurate feed consumption records being maintained throughout the 18-day experimental period.

The basal diet used is given in table 1. It is similar to the diet employed by Anderson et al. ('51) although differing in the following respects: 2 mg % of niacin were added to our diet since this was considered a marginal level which should not suffice to overcome the growth depression expected from 4% of glycine; instead of 5% gelatin we supplemented our diet with 1% each of L-arginine HCl and glycine. Vitamin B_{12} was omitted from the vitamin supplement.

TABLE 1

Basal diets used in experiments 1 and 2

INGREDIENTS	EXP. 1 AMOUNT	EXP. 2 AMOUNT	VITAMINS ADDED	AMOUNT
	%	%		*mg/kg*
Casein (Vitamin test)	18.00	....	Thiamine HCl	25
Drackett protein [1]	....	30.00	Riboflavin	16
Corn oil	3.00	3.00	Calcium pantothenate	20
Salts (Glista, '51) [2]	5.34	5.34	Niacin	20
DL-methionine	0.30	....	Pyridoxine HCl	6
Roughage [3]	3.00	3.00	Biotin	0.6
Choline Cl	0.20	0.20	Folic acid	4
L-cystine	....	0.25	Inositol	100
L-arginine HCl	1.00	....	Para-amino benzoic acid	2
Glycine	1.00	....	Menadione	5
Glucose [4]	68.16	58.21	α-tocopherol acetate	20
Vitamin B_{12}	....	4 μg	Ascorbic acid	250
Niacin	....	20 mg	A & D (10,000 A–600 D_3)	1000
Vitamins	+	+		

[1] The 30% added is approximately equivalent to 25% protein on a dry basis.

[2] Composition of salt mixture may also be found in the paper by Fisher et al. ('54a).

[3] Ruffex.

[4] Cerelose.

In table 2 are shown the growth and feed utilization data for experiment 1. It is apparent that glycine did not produce any toxic or growth-depressing effect. The slightly better growth with supplemental niacin or vitamin B_{12} which, however, was not statistically significant, indicates that the basal diet was sufficiently low in these nutrients. On the other hand, glycine significantly ($P < 0.0001$) improved growth and

markedly reduced the variability among the chicks of the same group.

While these results were unexpected, it was still more surprising to observe the remarkable effect of glycine on feed utilization. It can be seen in table 2 that the additon of glycine to the diet, in each case, improved the ratio of gain to feed consumed. These differences are highly significant by Student's t test for paired data ($P < 0.0001$).

TABLE 2

The effect of excess glycine supplementation on the growth and feed utilization of chicks

LOT NO.	SUPPLEMENT TO BASAL	AVERAGE 25-DAY WEIGHTS	CUMULATIVE 7 — 25-DAY RATIOS
		gm	*gm gain/gm feed*
1	None	269 [1]	0.461
2	4% glycine	314	0.658
3	B_{12}, 40 μg/kg	303	0.507
4	As in 2 +3	307	0.711
5	Niacin, 200 mg/kg	285	0.498
6	As in 2 + 5	288	0.595
7	Niacin + B_{12}	297	0.515
8	As in 2 + 7	315	0.672

[1] Average of 10 chicks per lot.

INVESTIGATIONS ON THE MODE OF ACTION OF GLYCINE

In view of the interesting observation that excess glycine added to diets already containing the 1 to 1.5% glycine postulated by Almquist to be required by the chick, not only did not depress growth, but actually improved feed utilization significantly, the following experiment was designed to elucidate these findings. Fisher and Scott ('54) have discussed recent evidence for a much higher arginine requirement for the chick than had previously been proposed by Almquist ('50). Since we had included 1% arginine in our present diet, it appeared possible that the glycine toxicity observed by other workers might have been due to a low arginine level in their diets (according to the present estimate of requirement of 1.7% arginine, most of the reports in the literature concerned with chickens showed diets low in this amino acid).

This was also the main difference between our diet and the one used by Anderson et al. ('51). Our hypothesis implied that (1) an amino acid imbalance was created due to the inadequacy of an essential amino acid and that (2) niacin, vitamin B_{12} and folic acid might overcome this toxicity by a non-specific removal of the excess dietary amino acid. This hypothesis was supported by the work of Russell et al. ('52) who showed that excess essential amino acids did not depress growth of the white rat when the diet contained the levels of essential amino acids recommended for the rat by Rose. Only methionine and lysine exerted a growth depression and for these amino acids it was concluded that it was a property of the compound rather than an amino acid imbalance.

To test the validity of our hypothesis, three different amino acid imbalances were designed. In the first, the protein casein was used with suboptimal arginine supplementation and 4% glycine as the unbalancing amino acid. The second case, similar to the first, employed 4% DL-methionine as the unbalancing amino acid. The third condition was created by using soybean-protein which is presumably low in methionine for the chick, with glycine as the unbalancing amino acid. The design employed in experiment 2 and just outlined is given in table 3.

The casein basal diet in this experiment was identical to the one used in experiment 1 (table 1), except that 0.4% L-arginine HCl was added instead of 1% in order to have the basal diet deficient with respect to this amino acid. The composition of the isolated soybean-protein (Drackett) diet is also given in table 1. All excess amino acids were added at the 4% level.

Female day-old chicks of the NH ♂ × Columbian ♀ mating were placed in group batteries and one group of 200 chicks was fed the basal casein diet, while 100 chicks were fed the soybean (Drackett) basal diet for one week. At the end of the week, the chicks on their respective basal diets were allotted by weight into the 12 different groups of 15 chicks each, and were fed the diets outlined in table 3. Accurate

TABLE 3

The effect of glycine on chick growth and feed utilization as influenced by amino acid balance

LOT NO.	PROTEIN SOURCE	DEFICIENT AMINO ACID	UNBALANCING AMINO ACID	OTHER CONDITIONS	AV. 26-DAY WTS.	CUM. 7 — 26-DAY RATIOS
					gm	*gm gain/gm feed*
1	Casein	Arginine	....	B_{12} free, low niacin	237 [1]	0.443
2	Casein	....	....	B_{12} free, low niacin	291	0.525
3	Casein	Arginine	Glycine	B_{12} free, low niacin	276	0.559
4	Casein	....	Glycine	B_{12} free, low niacin	305	0.605
5	Casein	Arginine	Glycine	B_{12} added, low niacin	293	0.543
6	Casein	Arginine	Glycine	B_{12} free, niacin added	285	0.558
7	Casein	Arginine	Methionine		67 [2]	.... [3]
8	Casein	....	Methionine		72 [2]	.... [3]
9	Soybean [4]	....	....		365	0.571
10	Soybean	....	Glycine		369	0.622
11	Soybean	Methionine	....		340	0.547
12	Soybean	Methionine	Glycine		350	0.681

[1] Average of 15 chicks per lot.

[2] These chicks were taken off experiment at 21 days of age.

[3] Ratios for these groups are not given since the birds did not gain any weight.

[4] Drackett protein.

weekly weight and feed consumption records were kept for the duration of the experiment.

Table 3 gives the final weight and feed utilization data. With the exception of lots 7 and 8, which received the 4% excess methionine in their diets, all chicks were carried to 26 days of age; in the case of lots 7 and 8 the birds were taken off experiment at 21 days of age.

It was already apparent at the two-week weighing that our hypothesis concerning growth depression due to amino acid imbalance did not adequately explain our previous and present findings. Thus, as before, glycine did not depress growth under any of the conditions tested, either on the casein or the Drackett protein diet. Methionine depressed growth sharply but this was not overcome by a higher level of arginine in the diet. The work by Russell et al. ('52) was not known to us at the beginning of this experiment, thus, methionine was probably a poor choice as an amino acid with which to produce an imbalance since its depressing effect appears to be of a pharmacological nature.

Statistical analysis of the data in lots 1 through 4 shows a significant arginine effect ($P < 0.05$) demonstrating again the inadequacy of 1.2% of this amino acid for optimum chick growth. Besides the poorer growth and the extreme variability in weight, feather development was also markedly retarded on the arginine-low diet. While glycine, vitamin B_{12} and niacin supplementation slightly improved the rate of growth (not significantly, however) the poor feathering of these birds nevertheless indicates the insufficiency of arginine in these diets. Methionine supplementation did not improve growth on the Drackett protein diet even though this diet contained only 0.3% methionine. Added methionine might have improved growth somewhat since even the lowest estimates of the methionine requirement of the chick by Glista ('51) and Griminger and Scott ('54) have shown its requirement to be about 0.34% in the presence of adequate cystine.

Again we must point to the striking effect of glycine on the feed utilization ratio. This is particularly evident for

lots 9 and 10 and lots 11 and 12 which can be considered as pairs with identical growth permitting the best comparison of the ratios of gain to feed consumed. The glycine effect is again statistically highly significant ($P < 0.02$).

After the first week on experiment, it was apparent that we had failed to obtain glycine toxicity but had repeated our earlier findings of the effect of glycine on the improvement of feed utilization, this time with yet another protein source. Because of the important implications of these results an attempt was made to account for this action of glycine.

Three major functions of glycine are known: (1) it is a precursor of uric acid (Buchanan et al., '48); (2) it is a precursor of creatine (Bloch and Schoenheimer, '40); (3) it is an important constituent of feather protein (Block, '39).

It was considered of interest, therefore, to determine the feather content and muscle creatine level of chicks raised with and without dietary glycine supplementation.

In a preliminary test, we removed and sacrificed three birds each from lots 11 and 12 (see table 3 for design) after they had been on the experiment for 10 days. Since there were no visible differences in feathering such as had been observed on the low arginine diets, we carefully removed all feathers and pin feathers and weighed them to the nearest 0.1 gm in order to see if there were any differences in total feather content. We also measured the length of all the long wing feathers. After weighing and measuring, the feathers were ground in a Wiley mill and glycine was determined microbiologically with *Leuconostoc mesenteroides* P-60 as the assay organism.[3]

Leg muscle creatine was determined (as total creatinine) by the method of Rose et al. ('27) which Hegsted et al. ('41) had found very reliable for their work. Duplicate tissue samples were taken from each bird to ascertain the reliability of the method.

The feather and muscle creatine data are given in table 4. Statistical analysis showed that glycine supplementation had

[3] We wish to thank Mrs. Carol Promislow for running these assays.

TABLE 4

The effect of glycine feeding on feathering and muscle creatine

17-day-old chicks

CHICK NO.	GLYCINE ADDED	FEATHER WEIGHT	FEATHER LENGTH	GLYCINE CONTENT OF FEATHERS	MUSCLE CREATINE	
					Rep. 1	Rep. 2
			cm	%		
4363	+	4.12 [1]	8.6 [2]	5.6	1.154 [3]	1.249 [3]
4371	+	4.47	8.8	6.4	1.305	1.232
4362	+	4.98	9.3	4.8	1.251	1.262
Average of group		4.52	8.9	5.6	1.242	
4348	—	3.61	8.7	6.4	1.108	1.127
4355	—	3.85	8.5	7.6	1.111	1.082
4347	—	3.38	8.1	5.6	1.136	1.134
Average of group		3.61	8.4	6.5	1.116	

26-day-old chicks

CHICK NO.	LOT NO.	GLYCINE ADDED	FEATHER WEIGHT	MUSCLE CREATINE
4320	9	—	5.91 [1]	3.55 [3]
4314	9	—	6.08	3.66
NB	9	—	5.42	3.72
4318	9	—	4.41	3.34
4319	9	—	5.68	3.70
Average of group			5.50	3.59
4330	10	+	7.44	3.80
4335	10	+	7.85	3.68
4332	10	+	6.35	3.60
4341	10	+	5.69	3.98
4333	10	+	6.29	3.71
Average of group			6.72	3.75
4351	11	—	6.14	3.50
4353	11	—	4.42	3.15
4352	11	—	5.97	3.38
4356	11	—	4.63	3.67
4343	11	—	5.03	3.32
Average of group			5.24	3.40
4358	12	+	8.13	3.58
4368	12	+	6.39	3.78
4361	12	+	6.30	3.76
4364	12	+	6.65	3.54
4370	12	+	6.10	3.75
Average of group			6.71	3.68

[1] As a percentage of total live weight.

[2] Average length of the long wing feathers.

[3] Milligrams per gram of fresh tissue.

significantly increased the total feather content as a percentage of whole body weight ($P < 0.03$) as well as the level of muscle creatine ($P < 0.0001$). There were no differences in the length of the wing feathers or in the glycine content of the feathers.

At the end of the 26-day experimental period, 5 birds each were sacrificed from lots 9 through 12 and feather weight and muscle creatine again determined by the methods mentioned above. The results of these determinations are also listed in table 4. Again glycine supplementation significantly improved feathering ($P < 0.02$) and increased the level of muscle creatine ($P < 0.01$).

DISCUSSION AND RESULTS

Our inability to produce a glycine toxicity suggests the following: (1) Vitamin B_{12} does not appear to be involved in the toxicity syndrome except perhaps under conditions of severe B_{12} depletion which had been the case in the references cited. This suggestion had already been made by Naber et al. ('52). (2) The level of 2 mg % niacin included in our casein diet may be too high to permit the development of glycine toxicity, although this appears improbable since we did obtain a slight growth response from higher levels of niacin, and did not encounter any perosis due to glycine supplementation. (3) It may be that the presence of gelatin in the diet is essential in producing the glycine disturbance which has been noted by earlier workers.

The beneficial effect of glycine in improving feed utilization can be of great practical importance. In experiment 3, we tested the effect of 2 and 4% glycine supplementation to a practical corn-soybean chick ration. The results shown in table 5 indicate again an improvement in feed utilization and in growth with the 2% glycine that borders on statistical significance. It is interesting in this connection to quote from a report by Almquist ('42) to the effect that "addition of glycine to a practical chick starting mash of excellent quality caused noticeably faster growth." Since the average soy-

bean or fish meal type of chick ration normally contains at least 1 to 1.5% glycine, the beneficial effect described by Almquist may well have been of the same nature as the one observed in our studies.

The creatine and feather data offer a partial answer to the effectiveness of glycine in improving the feed utilization of chicks. Since the chick appears to be very inefficient in synthesizing glycine, at least during early growth and development, it is not unreasonable to expect nitrogen wastage in order to obtain the necessary dietary glycine for proper tissue and feather protein synthesis. In this respect it has

TABLE 5

Effect of glycine supplementation to a practical chick diet

LOT NO.	SUPPLEMENT TO BASAL [1]	4 WEEK WEIGHTS	CUMULATIVE 1 — 28-DAY RATIOS
		gm	*gm gain/gm feed*
1	None	454 [2]	0.533
2	2% glycine	496	0.554
3	4% glycine	446	0.549

[1] Percentage composition of basal diet: corn 55.78, soybean oil meal (50%) 34.00, bone meal 3.00, limestone 1.00, salt 0.5, A and D oil 0.05, $MnSO_4$ 0.05, DL-methionine 0.10, alfalfa 1.0, aurofac 0.5, riboflavin conc. 0.02, corn distillers solubles 4.0; niacin 1 gm and choline (25%) 96 gm/100 lb. were added.

[2] Average of 10 chicks per lot.

been shown by Ackerson and Blish ('25–'26) that the addition of cystine to the diet of molting hens markedly reduced the endogenous nitrogen loss. Besides the glycine which is present in the feather protein, it has also been shown that bird feathers contain 130 mg % of uric acid which would again represent a glycine requirement for its biosynthesis (Bollinger and Tow, '46).

The creatine data provide additional information to account for the better feed utilization of diets containing excess supplementary glycine. It is very interesting to note the very low creatine values in the leg muscles of 17-day-old as compared to 26-day-old chicks. A careful search of the literature has revealed that the muscle creatine content of various

animal species does not reach its maximum until some time after birth or hatching. Rose ('11) reported that the creatine content in the muscle of newborn infants was only 1.9 mg/gm of tissue (as compared to 3–4 mg/gm in adults). Rose ('11) and more recently Catherwood and Stearns ('37) showed that in infants the excretion of creatinine increases from birth to one year of age. Cohen ('51) has recently investigated the methylation of guanidoacetic acid to creatine in embryonic and adult tissues to offer a possible explanation of the low creatine content during early life. He found no differences in enzymatic activity. This, however, might be taken as indirect evidence that the formation of guanidoacetic acid itself may be the limiting one in early periods of growth. The high requirement for these amino acids can be explained on the basis of the chicks' low ability to synthesize its own arginine and glycine.

Mellanby ('08) has shown for the chick that there is a definite increase in muscle creatine content with age. In his studies maximum values for muscle creatine were reached by 14 days; however, it is important to note that his chicks (which had the same hatching weight as ours) weighed only 58 gm at two weeks while in our experiment they weighed close to 200 gm.

Mellanby further showed that dietary creatine or precursors had no effect on the muscle creatine level after the maximum had been reached. Before this time dietary creatine increased the muscle creatine content. In view of the extremely low creatine levels during the first weeks of development as shown by our 17-day analysis, the importance of glycine is accentuated.

Careful appraisal of our results suggest that the effect of glycine in producing either the toxicity experienced by other workers or the beneficial effect as found in our studies is closely related to the age and development of the birds, particularly with reference to their ability to synthesize their own glycine. Thus it is interesting to note that by 26 days the creatine levels had reached approximately normal values,

and that the effect of glycine supplementation had decreased as the creatine levels approached maximum values. Furthermore, the feed utilization ratios, given by weeks in table 6, show a definite decreased difference due to glycine supplementation with increasing age of the chicks. It may be conjectured that high dietary excesses of glycine may become toxic by the time the chick's ability to synthesize glycine has increased to such a degree that optimum levels of creatine are produced. This condition may also occur when the rate

TABLE 6

The decreasing influence of glycine supplementation on feed utilization with increasing age of the chick

LOT NO.	GLYCINE ADDED	RATIOS OF GRAMS GAINED PER GRAM OF FEED CONSUMED			
		7–14 days	14–21 days	21–26 days	Cumulative 7 — 26-day ratios
					gm gain/gm feed
1 [1]	—	.498	.423	.444	.443
3	+	.664	.529	.519	.559
2	—	.591	.513	.488	.525
4	+	.709	.606	.536	.605
9	—	.604	.597	.518	.571
10	+	.692	.643	.549	.622
11	—	.596	.524	.533	.547
12	+	.725	.597	.590	.681

[1] The lots are presented in pairs so that the only variable between them is the presence or absence of added glycine.

of growth of relatively young birds is so impaired by other dietary inadequacies that the rate of glycine and therefore creatine synthesis will be commensurate with the chick's rate of growth.

It appears, therefore, that the glycine requirement is not easily defined and that improvements in growth or feed utilization are realized with increasing glycine levels over a wider range than the 1.5% heretofore recommended by Almquist.

SUMMARY

The effect of 4% glycine supplementation to diets low in niacin and vitamin B_{12} was studied. Under the conditions of

our experiments, glycine did not depress growth when fed in excess of 4% to diets already containing at least 1 to 1.5%. Instead, it significantly improved feed utilization, a phenomenon which decreased with advancing age of the birds. This better efficiency could be explained by significant increases in the percentage of feathers and muscle creatine levels in the glycine-supplemented chicks. The importance of glycine for creatine synthesis is accentuated by the fact that the muscle creatine content in early chick life is extremely low. The rise in muscle creatine content with age may be related to the decreasing difference in feed utilization due to glycine supplementation. The glycine requirement for optimum growth and feed utilization of fast-growing birds is in excess of 1.5% both in purified as well as practical starting rations.

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NUTRITIONAL STATUS OF THE AGING [1]

III. SERUM ASCORBIC ACID AND INTAKE

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SIX FIGURES

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One of the nutrients which is usually given prominence in any study of nutritional status is ascorbic acid. Because of the relative ease and accuracy with which this substance can be determined an unusually large number of studies of ascorbic acid status is available. Load tests with determination of urinary excretion of a single large dose as well as plasma and white cell analyses for level of circulating ascorbic acid are generally used to determine the status of populations. Many intake records and estimates as well as experimental studies of blood levels and urinary excretion under controlled conditions have also appeared during the last 20 years.

Questions which have still to be answered, however, concern the magnitude of the optimum intake and plasma levels, the true significance to health of saturation of the tissues with ascorbic acid, the metabolic function of this vitamin, and the effects of sex, age, chronic disease, drugs and other stresses on its utilization.

[1] This was part of the Western Regional Cooperative Project, W–4, on nutritional status of population groups. It was supported in part by funds appropriated under the Research and Marketing Act of 1946. Effective cooperation was given by the Human Nutrition Research Branch of the United States Department of Agriculture, the United States Public Health Service, the California State Department of Public Health and the San Mateo County Department of Public Health and Welfare.

In the course of a study of 577 men and women over 50 years of age living in San Mateo county, California, serum ascorbic acid was determined, physical signs of deficiency, particularly in the gums and skin, were looked for and a 7-day diet record as well as a diet history was obtained. The chief object of the study was the assessment of the nutritional status of these aging participants and the establishment of any correlations which may exist between intake and status as shown by circulating levels and presence or absence of the stigmata of deficiency or excess.

Most of the ascorbic acid studies have dealt with children, college students and other young adults. Extensive evaluation of the ascorbic acid status of aging people has not been attempted. Criteria of deficiency have been various, but are usually based on a minimum value for plasma ascorbic acid. In a review (Nat. Res. Council Bull., '43) these criteria have been listed as ranging from 0.15 to 0.8 mg% with 0.3, 0.4 and 0.6 used more often than any others as indicative of depletion. However in only 5 of the 18 surveys listed were normal adults other than college students examined. In general population surveys the division has been usually between children and adults, all over 15 or 21 years of age being considered together as one group.

Kirk and Chieffi ('53) have reported whole blood ascorbic acid levels in 61 men and 81 women, 40 to 103 years of age living in an institution and receiving a diet which supplied 45 mg ascorbic acid per day. They noted a significant decline with age, $r = -0.44$, $t = 3.20$, in men but no significant change in women. The men had blood ascorbic acid levels of 0.59 to 0.33 mg% and the women 0.48 to 0.40. These blood levels are not inconsistent with the intake reported.

PROCEDURE

The participants were volunteers, all over 50 years of age, residents of San Mateo county and, in their own opinion, well. No participants were accepted who had sought medical advice within three months prior to the examination. No

effort was made to obtain a true sample of the county population since establishment of correlation between diet habits and physical conditions and results of biochemical analyses was the main purpose of the study. All of the participants lived in their own homes except for 44 men in the county home.

Each subject was brought to the laboratory trailer where a blood sample was taken by venipuncture. A part of the unoxalated sample was allowed to stand for 15 to 20 minutes, then was centrifuged for 15 to 20 minutes and the separated serum used for the ascorbic acid determination. The aliquots were acidified at once with trichloracetic acid and placed in the refrigerator at 0°C. until the test was completed on that or the following day. No loss occurs in this procedure, but large losses of ascorbic acid in serum may occur if there is delay in adding the trichloracetic acid. The method used for ascorbic acid was that of Lowry, Lopez and Bessey ('45).

The participants were given directions as to the content of the last meal to be eaten before reporting for the examination. This consisted of carbohydrate food chiefly, with little fat and no fruits or vegetables, and was eaten not less than two hours before the sample was drawn. Each subject was questioned on this point at the time of the examination and any deviations from the directions noted. Very few deviations except as to time of meal occurred. The 7-day diet records [2] were made at varying periods of one to 20 days previous to the physical examination.

These subjects were examined from October to May with some consequent variation in fresh fruit and vegetable intake. The differences were not large, however, because of the relatively constant and uniform supply of these foods in the local markets.

[2] The directions for keeping the diet records were given the participants by Clara Beth Young, June Baldwin, Madalyn R. Tomassetti and Helen W. Hubbard. Diet histories were also taken but these were not used in the calculations of intake except as a check on the probable accuracy of the records. The method of calculation of intakes was described previously (Gillum and Morgan, Nutritional Status of the Aging. I. Hemoglobin levels, per cent packed cell volumes and sedimentation rates. J. Nutrition, *55:* 265, 1955.

TABLE 1

Serum ascorbic acid levels and ascorbic acid intakes of men and women over 50 years of age

AGE	NO. OF SUBJECTS	BODY WT.	SERUM ASCORBIC ACID			"t" VALUE	DAILY ASCORBIC ACID INTAKE		
			Mean	Range	Standard error		Mean	Standard error	Mean per kg
yrs.		*kg*	*mg %*	*mg %*			*mg*		*mg*
50–54									
Men	45	74.1	0.73	0.11–1.72	0.06	4.02	97	8	1.3 ± 0.11
Women	49	68.2	1.10	0.10–1.88	0.07		84	8	1.2 ± 0.12
55–59									
Men	40	74.5	0.75	0.11–2.06	0.08	3.58	97	7	1.3 ± 0.09
Women	53	65.9	1.13	0.13–2.14	0.07		90	7	1.3 ± 0.10
60–64									
Men	36	74.5	0.94	0.33–1.44	0.06	2.70	136	11	1.8 ± 0.15
Women	57	65.4	1.21	0.07–2.50	0.08		91	6	1.4 ± 0.09
Co. Home men	11	66.4	0.27	0.05–0.72	0.06		44	6	0.7 ± 0.09
65–69									
Men	39	70.9	0.91	0.19–2.28	0.08	0.80	89	8	1.2 ± 0.10
Women	57	65.0	0.99	0.09–2.14	0.06		88	5	1.3 ± 0.08
Co. Home men	16	61.8	0.29	0.07–1.16	0.06		41	8	0.7 ± 0.13
70–74									
Men	36	70.9	0.76	0.13–1.96	0.09	1.91	93	9	1.3 ± 0.13
Women	40	63.6	0.99	0.20–1.82	0.08		89	8	1.4 ± 0.12
Co. Home men	8	67.7	0.25	0.10–0.66	0.06		35	2	0.5 ± 0.03
75 plus									
Men	36	70.5	0.92	0.19–1.96	0.15	0.12	97	18	1.4 ± 0.25
Women	37	63.2	0.95	0.13–2.53	0.18		76	20	1.2 ± 0.32
Co. Home men	9	62.2	0.22	0.09–0.24	0.06		36	8	0.6 ± 0.13
Totals									
men	232	72.4	0.83	0.11–2.28	0.08	2.2	99	10	1.36 ± 0.14
Women	293	67.1	1.07	0.07–2.53	0.08		86	8	1.28 ± 0.12
Co. Home men	44	64.1	0.27	0.05–1.16	0.06		40	7	0.62 ± 0.10

RESULTS

The mean serum ascorbic acid levels are shown in table 1, segregated by age and sex. It is obvious that the mean values for each age group of women were higher than the mean values for men of the corresponding age groups. The differences were significant except in the 65 to 69 and 75 + age groups. In both sexes there was noted a high point at 60 to

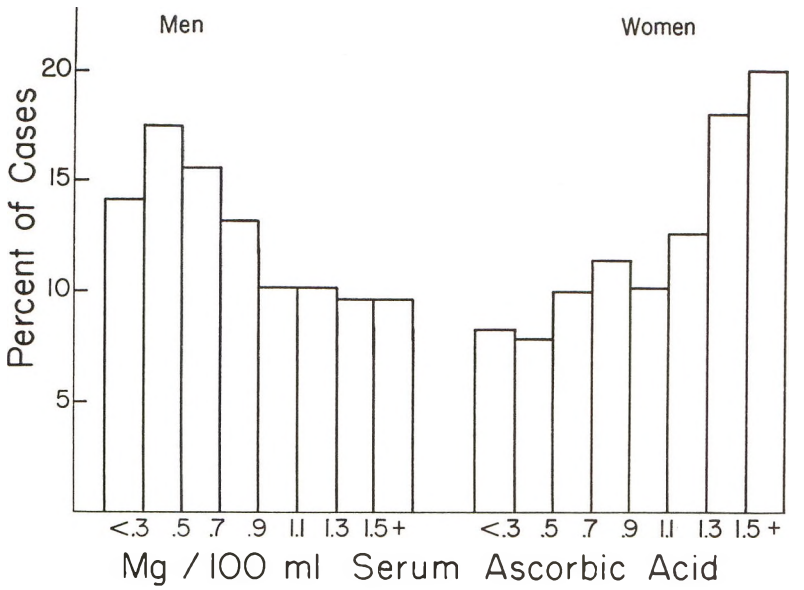

Fig. 1 Distribution of serum ascorbic acid of 232 men and 293 women living in their own homes.

64 years. Very low values were found in the 44 men living in the county home. The distribution of these levels for men and women living in their own homes is shown in figure 1. The distribution curves are reversed, that of the men being skewed to the left and that of the women equally to the right.

Intakes

The ascorbic acid intake records are shown in table 1 and their distribution in figure 2. In all of the age groups the

total intakes of the men were larger than those of the women although they were significantly larger in only the 60- to 64-year-old group. The intakes of the men in the county home were low, usually less than one-half that of the others, but these are not included in figure 2. The distribution of intakes of the women approaches the normal curve, but that of the men is less regular. The peak of intake at age 60 to

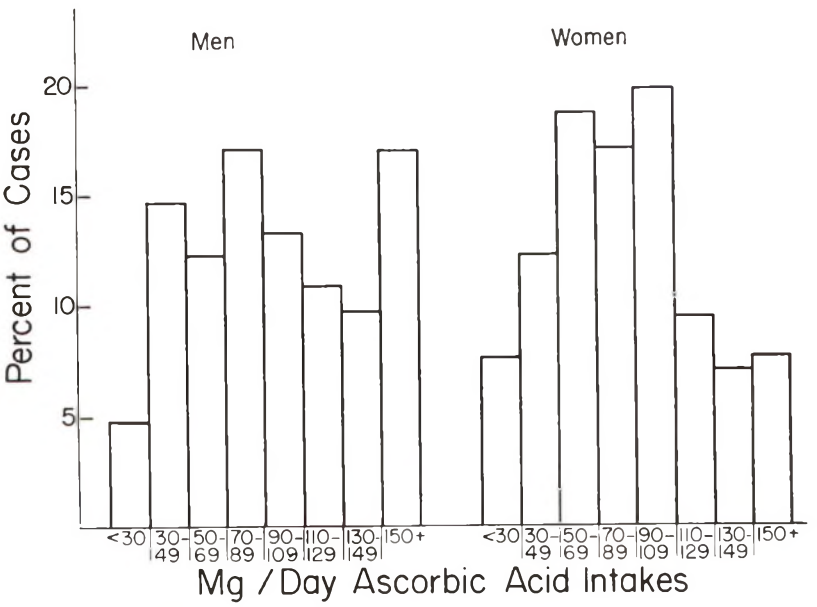

Fig. 2 Distribution of daily ascorbic acid intakes of 232 men and 293 women living in their own homes.

64 years in the men living in their own homes is surprising but is apparently not an artefact. Sixty per cent of the men in this group had an intake of more than 150 mg per day and only two of these took ascorbic acid tablets.

When the daily intakes were expressed as milligrams of ascorbic acid per kilogram body weight (table 1) the differences between the intakes of men and women living in their own homes tended to disappear. On this basis the means of the entire groups were found to be nearly identical. The in-

take of the men in the county home on this basis also was slightly less than half this value.

The higher metabolic rate of men per kilogram of body weight, due in part to their greater proportion of lean body mass, may account for a correspondingly greater ascorbic acid requirement. However, when the intakes shown in table 1 were recalculated so as to increase the ascorbic acid intake of the women by 10%, to correspond with the usually accepted lower basal metabolic rate of women, no significant differences emerged. The intakes of the men were greater than or not significantly less than those of the women even with this correction.

Correlation of serum levels and intakes

The possible correlation of serum ascorbic acid levels with intakes was tested by dividing the values for men into three groups of roughly similar magnitude, those with serum levels of, (a) less than 0.5 mg%, (b) from 0.5 to 1.09, and (c) more than 1.09. A similar division of the values for the women was, (a) less than 0.7 mg%, (b) from 0.7 to 1.29, and (c) more than 1.29. The different division of serum level values for men and women was adopted in order to obtain roughly equal groups for each range of these values. Even when the serum values for the 44 men in the county home were removed from this grouping of the men, the division still fell in ranges significantly lower than those for the women. The average daily intakes of men and women were also divided into three groups, (a) less than 70 mg, (b) 70 to 109 and (c) more than 109. The proportions of persons consuming each of these three amounts of ascorbic acid in each of the three serum ascorbic acid groupings were then calculated and are shown in figure 3. It is obvious that 70 to 75% of both men and women in the lowest serum ascorbic acid groups had the lowest level of intake, with only small numbers, 22 and 13% in the middle intake group and 5 and 11% in the highest intake group. Similarly in the middle range of serum levels

nearly half of both men and women were in the middle intake groups. In the highest, as compared with the lowest serum level groups, the percentage was reversed, that is, one-half to two-thirds were in the highest intake group and smaller

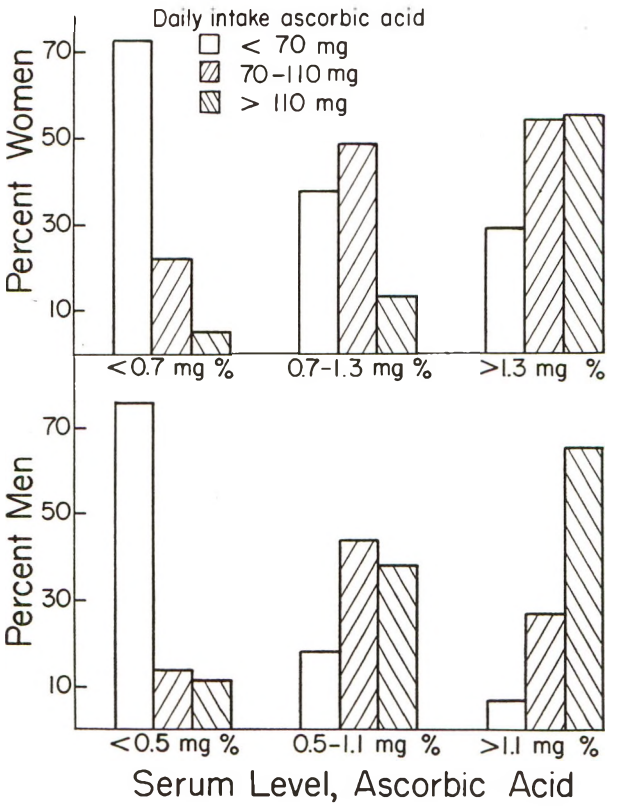

Fig. 3 Distribution of ascorbic acid intakes of women having serum ascorbic acid levels less than 0.7, 0.7 to 1.3, or more than 1.3 mg% and men having 0.5, 0.5 to 1.1 or over 1.1 mg%.

numbers, 29% for women and 6% for men, in the lowest intake groups. The correlation is more sharply defined in the men than in the women. The correlation statistic r for serum and dietary ascorbic acid for the entire group was + 0.46, significant at the 1% level.

Conversely the predictability of serum levels from daily intakes was tested. As shown in figure 4 the percentage of persons with the lowest serum levels is highest in the lowest intake group, intermediate in the middle intake group and low in the highest intake group. Again the correlation in the values for the men is more marked than in those for the women.

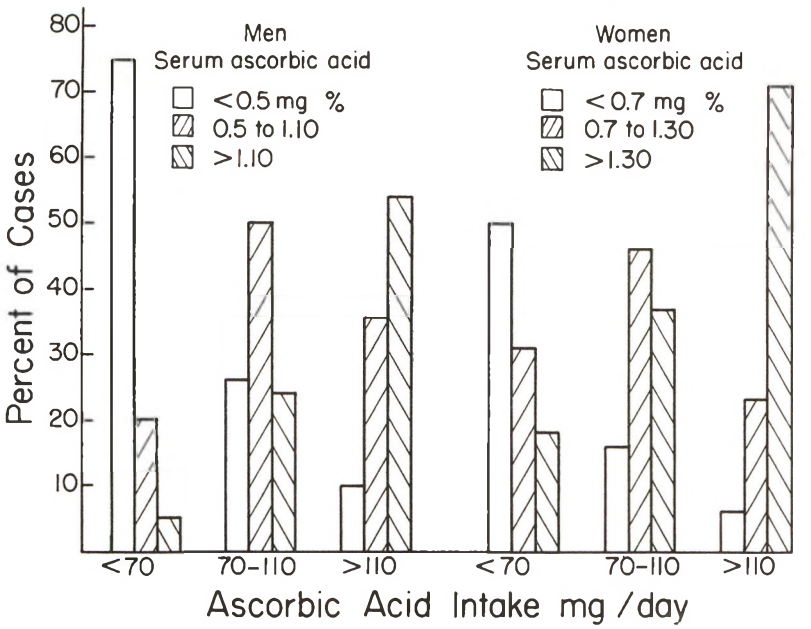

Fig. 4 Distribution of serum ascorbic acid levels of men and women who reported daily ascorbic acid intakes of less than 70, 70 to 110 or more than 110 mg.

Relationship to socioeconomic level

An interesting relationship of the socioeconomic level with the serum ascorbic acid values was seen when the subjects were separated into 4 income groups, based on the judgment of the interviewers.[3] These were, (a) men in the county home, 44 included, (b) men and women known to be receiving pen-

[3] This comparison was suggested in a preliminary paper by Chope and Dray ('51).

sions or county relief funds or although not on public assistance rolls obviously with very small income, 102, (c) the middle income group, 396, and (d) those with very high income, 23 persons. The total was 566 rather than 577, the

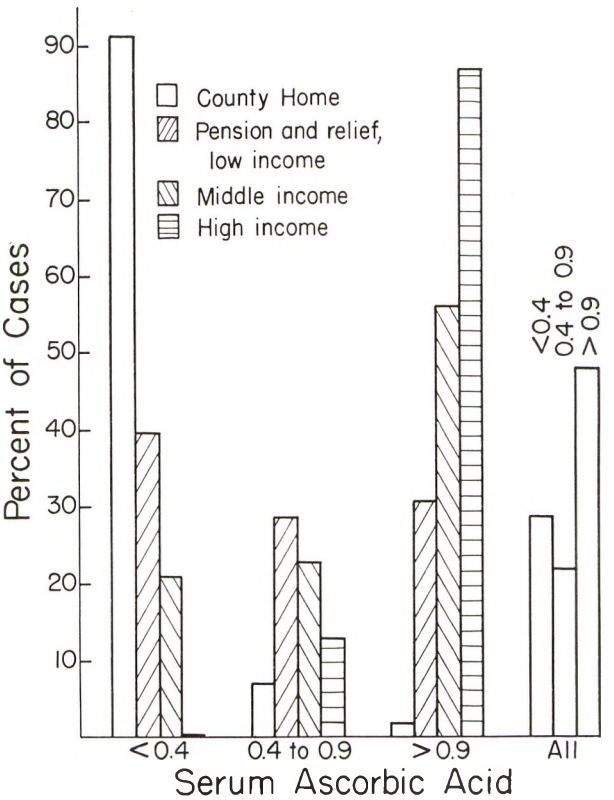

Fig. 5 Relationship of economic levels with mean serum ascorbic acid levels.

maximum number enrolled, since serum levels were not available for 11 of the original subjects. Each of these 4 groups was sorted for serum ascorbic acid levels into three groups with levels of less than 0.4 mg%, 0.4 to 0.9, and more than 0.9. As shown in figure 5 the two lower income level groups had a much larger percentage of low serum values than did the other two groups and the reverse was true of the high

serum values. Bessey and Lowry ('45) reported a similar distribution of plasma ascorbic acid levels in three groups of a New York high school survey of children of different economic levels.

It is interesting to note that the people on relief and very low incomes maintained considerably better serum levels than did the men in the county home. The low intakes of the latter group appeared to result, at least in part, from the institutional use of long cooking processes and steam tables, since the serum levels were lower than the calculated value of the diets would seem to justify. Dodds et al. ('50) found that 24 young women subjects on prolonged intake of about 56 mg ascorbic acid a day attained blood plasma ascorbic acid equilibrium levels of 0.79 mg%. However, Haines et al. ('47) found plasma levels of 0.28 to 0.36 mg% of ascorbic acid for two men and two women, 19 to 29 years old, after 6 weeks on a daily intake of 33 mg. The difference in age and sex between these subjects and the old men in the county home who had a daily intake of 35 to 44 mg would tend to favor lower values in these men but probably not the extremely low figures, average 0.27 mg%, observed. Their true intake level may have been lower than the calculated mean, 40 mg.

The amount and kind of dietary supplements taken by the subjects were recorded by the interviewers. It was found that 24% of the men and 32% of the women took more or less regularly some such supplements. Of these, 39 men and 51 women took multivitamin preparations, some of which also contained minerals, and three men and one women took ascorbic acid tablets. Three men took vitamins A and D mixtures and 24 men and 33 women vitamin B complex preparations. Thus, 42 men and 52 women may have received ascorbic acid in addition to that present in their food. There was no concentration of these subjects in any one age group. None of these additions were included in the calculations of nutrient intakes.

Occurrence of abnormal gum conditions

The most frequently mentioned physical sign associated with ascorbic acid deficiency is so-called gingivitis. Reddening, edema, sponginess, retraction, suppuration or bleeding of gums are included under this name. Hanke ('30) observed significant amelioration of these conditions when the subjects were given liberal amounts of citrus juice, raw cabbage or lettuce. Kyhos et al. ('44) in a study of 71 male prisoners likewise noted remarkable prevalence of oral diseases and loss of teeth correlated with very low levels of plasma ascorbic acid. Youmans et al. ('45) found "gingivitis" or "pyorrhea" in 49 and 55% of white and negro males over 21 years of age and in 32 and 48% of white and negro females of the same age range. A significant correlation was found between the occurrence of this sign and the prevalence of low serum ascorbic acid levels. Of 551 persons in this study for whom records [4] of oral condition were considered, 219 or 39% were edentulous, 94 or 17% had gingivitis of some degree, mostly moderate, and 238 or 43% had relatively healthy oral tissues. Of the endentulous 24 had serum ascorbic acid levels below 0.3 mg%, of those with gingivitis 23% had similarly low serum levels but of those with teeth and no gingivitis only 9% had serum levels of this order. Conversely, considering all those who had serum ascorbic acid levels below 0.3 mg%, 17% of all examined, 55% were edentulous, 22% had gingivitis and 22% had no gingivitis.

If the serum level of 0.7 mg% be chosen as the line of demarcation, it will be found that of those with levels below this amount 47% were edentulous, 24% had gingivitis and 29% had healthy oral tissues. Among those with serum levels above 0.7 mg% 34% were edentulous, 12% had gingivitis and 54% had healthy gums.

In order to depict these relations more clearly all the subjects were divided into three groups, those with serum as-

[4] The physical examinations, including that of the oral tissues, were done by Dr. Sheldon Dray, United States Public Health Service.

corbic acid levels, (a) between 0.0 and 0.49 mg%, (b) between 0.5 and 1.09, and (c) over 1.09. These values were chosen because they assembled groups of similar magnitude, 161 persons in group a, 179 in group b and 211 in group c. The

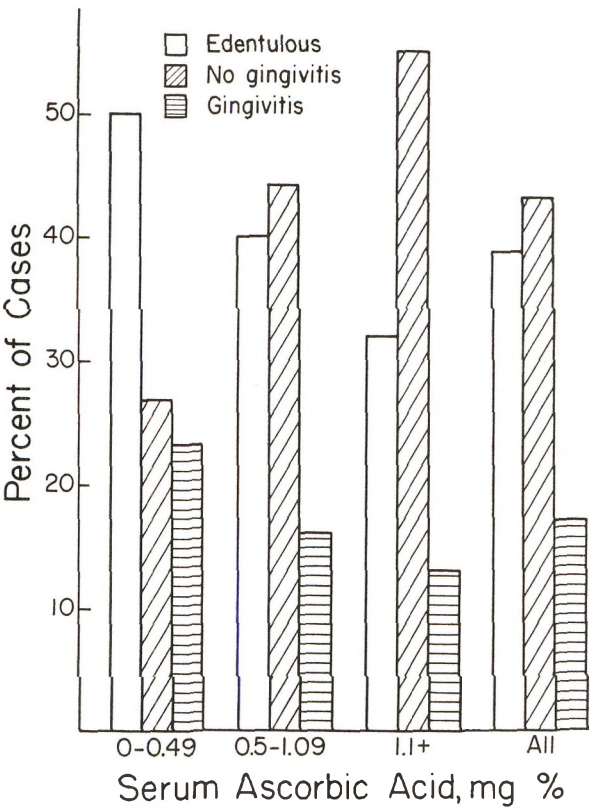

Fig. 6 Distribution of subjects without teeth and with or without signs of gum pathology in relation to serum ascorbic acid levels.

proportions of each group that were edentulous, that were judged to have some degree of gingivitis and that had no gingivitis, are shown in figure 6. It may be seen that the percentage of both the edentulous and those with gingivitis is considerably greater in the lowest serum level group, 0.0 to 0.49 mg%, than in the highest serum level group, over

1.09 mg%. The reverse is true of the "no gingivitis" group. The division in the middle serum level, 0.5 to 1.09 mg%, follows very closely the distribution in the entire population examined.

The data are presented for men and women together since the occurrence of these oral defects showed no sex difference. Twenty-one per cent of the men and 13% of the women had some degree of gum pathology, 43% of both men and women had no oral pathology and 36% of the men and 44% of the women were edentulous.

DISCUSSION

The statement is often made in discussion of the nutriture of old people that the tissues and fluids are low in ascorbic acid. Rafsky and Newman ('41) studied ascorbic acid retention in 25 so-called normal aged men and women. With oral doses of ascorbic acid up to 1000 mg only two subjects showed a fairly constant retention, 7 showed increasing retention with increasing dosage and in 16 retention increased to a maximum and then decreased. This was interpreted to indicate a low degree of tissue saturation in most of the subjects. In another report low ascorbic acid levels were found in old people consuming the same diet which supported normal levels in young adults, (Westergaard, '40). From these and other studies the conclusion has often been drawn that low serum ascorbic acid in the aged may be due to a physiological mechanism.

There is some experimental support for the theory that age and sex affect ascorbic acid utilization. Dumm and Ralli ('49), who measured the urinary excretion of this vitamin in rats 30 to 103 days old, found increasing output in the older animals up to attainment of sexual maturity with a decline thereafter. This decline however was greater in the females than in the males. Adrenalectomized rats excreted less ascorbic acid at all ages than did the intact animals and also showed little change in excretion with age. The ob-

servations were interpreted to signify an important role of both the adrenal gland and the sex glands in the synthesis or utilization of ascorbic acid. Whether this conclusion is accepted or not, it is clear that some effect of both age and sex on the production and utilization of this vitamin is indicated. In most of the surveys of populations serum ascorbic acid levels for females have been reported as higher than those of the males and this has either been ignored or ascribed to greater intake by the females. In Tennessee (Youmans et al., '45) a smaller percentage of females than males had levels below 0.3 mg% in all but one of the age groups. These were mostly children and young adults. In Oregon large numbers of 14, 15 and 16 year old native born and reared boys and girls were compared. Serum ascorbic acid levels less the 0.6 mg% occurred in a considerably larger percentage of boys than girls and the reverse was true of the levels above 0.7 mg% (Storvick et al., '51).

In the data for the North Carolina populations so thoroughly studied by Putnam et al. ('49) the mean plasma ascorbic acid levels of the females over 15 years of age were significantly greater than those of the males of the same age group although the ascorbic acid intakes of the males were slightly but not significantly greater.

The total intake of ascorbic acid by the women in our study was at all ages lower than that of the men, yet their serum ascorbic acid concentration at all ages was higher. Even when the intakes were estimated per kilogram of body weight no advantage in the intake of the females was seen.

The rise in serum ascorbic acid in both women and men between 60 and 64 years of age may result from abrupt postmenopausal reduction in gonadotropic activity. Erb and Andrews ('42) noted a decrease in plasma ascorbic acid levels in bulls and cows when the gonadotropic substance of pregnant mare's serum was administered. Some relationship between sex hormone production and circulating ascorbic acid was also noted in the bovine species by Phillips et al. ('41).

SUMMARY

The serum ascorbic acid levels, 7-day records of ascorbic acid intake and physical condition, particularly of teeth and gums, were obtained for 569 supposedly healthy men and women over 50 years of age. All of these were living in their own homes except for 44 men over 60 years old living in the county home. The serum levels and daily intake values were grouped for men and women separately in 5 year age groups from 50 to more than 80 years of age.

The women at all ages had higher serum ascorbic acid levels than the men, means 1.07 ± 0.08 and 0.83 ± 0.08 mg% respectively. In both sexes a maximum was noted in the age group 60 to 64 years. The total daily intakes of the men however were greater at all ages, means 99 ± 0.10 mg for the men and 86 ± 0.08 for the women. The daily intakes per kilogram of body weight were 1.36 ± 0.14 and 1.28 ± 0.12 mg. Expressed thus the difference in intakes was not significant.

The 44 men in the county home had only 0.27 ± 0.06 mg% serum ascorbic acid and 40 mg total daily intake or 0.62 mg per kilogram of body weight.

A direct correlation, $r = +0.46$, was found between the serum levels and intakes of the whole group. Seventy to 75% of all men and women having less than 0.5 or 0.7 mg% of serum ascorbic acid respectively were in the lowest intake group with less than 69 mg of ascorbic acid in their daily food. Conversely 75% of the men and 50% of the women with intakes of this lowest level had serum ascorbic acid levels also in the lowest brackets.

An approximation of economic level was recorded with 44 men in the county home in one group, those on relief and old age assistance rolls in a second group, total 146 persons, the middle income group of 396 persons in another and those in the highest income group, 23 persons, in a 4th group. The mean serum ascorbic acid levels of these 4 groups progressed from low to high in this same order. Ninety-one per cent of the men in the county home, 40% of those on relief and old age pensions, 21% of those with the middle incomes and none

of those with the high incomes had serum levels less than 0.5 mg%.

Vitamin supplements which might have contained ascorbic acid were taken more or less regularly by 18% of the men and 11% of the women but these additions were not included in the intake calculations.

Thirty-nine per cent of the people examined were edentulous, 17% had gingivitis in some degree and 43% had teeth and no gum pathology. There was a consistently greater proportion, 24% of the edentulous and of those with gingivitis in the group with serum ascorbic acid below 0.3 mg% than of those with healthy mouths, 9%. A higher proportion of those with teeth and healthy gums, 54%, was found in the group with the higher serum levels, over 0.7 mg%. Thirty-four per cent were edentulous and 12% had gingivitis in this group.

The ascorbic acid requirement of men beyond 50 years of age appears to be significantly greater than that of women.

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NUTRITIONAL STATUS OF THE AGING [1]

IV. SERUM CHOLESTEROL AND DIET

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SEVEN FIGURES

(Received for publication September 25, 1954)

In a study of supposedly normal men and women over 50 years of age serum cholesterol levels, both total and free, were determined in 573 of them along with food intakes in a 7-day dietary record. The methods and objects of the study have been described (Gillum and Morgan, '55). Much attention has been focussed in recent years on serum cholesterol levels, particularly in aging persons because of their possible relationship to the development of atherosclerosis. Several reviews have been published recently (Anon, '53; Gofman, '52a; Keys, '52). Correlations between serum cholesterol levels and physical measurements, such as body weight and blood pressure, and dietary intakes, particularly of fat, cholesterol and protein were sought in the study here reported.

[1] This study was part of the Western Regional Research Project, W-4 on nutritional status of population groups. It was financed in part by funds appropriated under the Research and Marketing Act of 1946. Substantial help and cooperation were received from the Human Nutrition Research Branch of the United States Department of Agriculture, the United States Public Health Service, The California State Department of Public Health and the San Mateo County Department of Public Health and Welfare.

PROCEDURE

The method of Schoenheimer and Sperry ('34) was used to determine both free and total cholesterol in the serum. This method, used on 1 ml serum, covers concentrations from 110 to 700 mg% total and 45 to 300 mg% free cholesterol. For values above these maxima 0.5 ml serum could be used but for those below the minima no good adaptation was available. The extremes for total cholesterol encountered in our samples were 106 to 720 but there was only one value, 720, above 478 and only one, 106, below 136 mg%.

An unoxalated blood sample was obtained from each subject by venipuncture and about 12 ml of it centrifuged for 15 to 20 minutes after it had stood about 20 minutes. The serum was frozen at once and kept frozen until the cholesterol analysis was done.

RESULTS AND DISCUSSION

The mean values for total serum cholesterol of all the subjects separated by sex and 5-year age intervals are shown in table 1. All of the subjects were men and women living in their own homes with the exception of 43 men, over 60 years of age, living in the county home. The values for the latter group are shown separately. It is obvious that the women at all ages up to 75 years had significantly higher serum cholesterol than the men and that the men in the county home had levels significantly lower than those of the men living in their own homes. The means for the men and women of all ages living at home were 241 ± 8 and 270 ± 8 respectively and for the men in the county home 209 ± 12 mg%. The distribution of these values for men and women is shown in figure 1. More than half of the men had levels under 240 mg% but only 30% of the women had such low levels. Half of the women and 71% of the men had levels under 260. Up to 280 mg% there was a fairly constant difference of 20% between the proportions of men and women having cholesterol concentrations at or below a given level but above 280

mg% this difference decreased. It is clear that so-called hypercholesteremia must be defined separately for men and women. For example, only 7% of the men had values over 300 mg% but 22% of the women had values over that amount.

TABLE 1

Mean serum cholesterol of men and women over 50 years of age

AGE GROUP	NO. OF CASES	SERUM CHOLESTEROL LEVELS			
		Mean	Range	Standard error	Free cholesterol
years		*mg %*	*mg %*		*% of total*
50–54					
Men	45	252	166–357	7	27
Women	49	267	182–720	11	28
55–59					
Men	41	242	136–336	7	28
Women	56	257	172–425	6	27
60–64					
Men	36	239	149–340	7	27
Women	57	274	200–377	6	28
County Home men [1]	11	226	152–287	14	28
65–69					
Men	39	243	182–390	7	27
Women	57	275	170–390	6	28
County Home men	16	211	148–278	10	27
70–74					
Men	36	236	178–284	6	27
Women	40	273	186–438	8	28
County Home men	7	199	167–221	7	28
75–79					
Men	19	242	165–344	11	28
Women	25	257	140–336	9	28
County Home men	5	182	157–211	11	28
80 +					
Men	18	216	106–305	10	28
Women	12	236	157–286	11	28
County Home men	4	204	146–254	27	27
Totals					
Men	234	241	106–390	8	27.4
Women	296	270	140–438	8	27.9
County Home men	43	209	146–287	12	27.6

[1] Men in county home.

This difference cannot be ascribed to food intake since the intakes by the men of cholesterol, fat and protein, all of which were found to exert some positive effect upon the serum cholesterol, were in all cases equal to or greater than those of the women and likewise greater in the younger than in the older groups of both sexes (table 2). The inclination of the age-serum cholesterol curves may be related however to steroid hormone production.

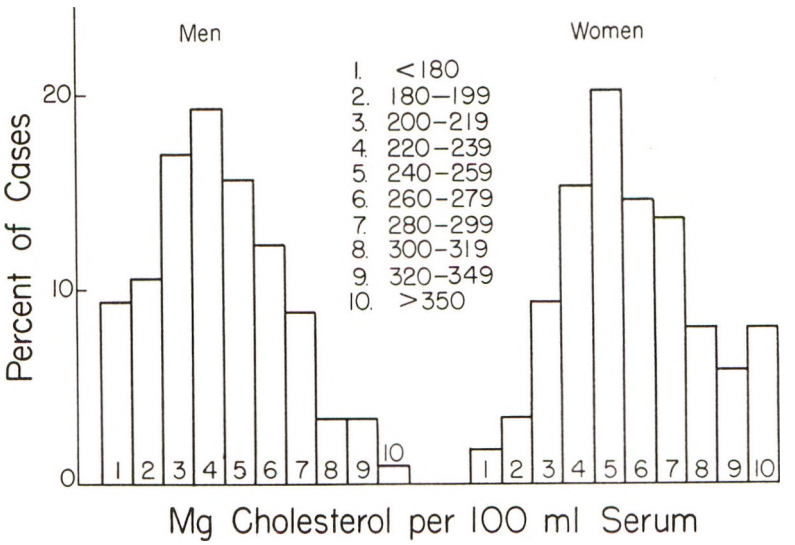

Fig. 1 Distribution of serum cholesterol levels of men and women over 50 years of age.

It is well known that excessive production of the follicle-stimulating hormone may occur in the female for one to 5 years following menopause. Progesterone production fails when the lutenizing hormone is no longer produced by the anterior pituitary and estrogens cease to be manufactured or circulated by the ovary but the monophasic gonadotropin continues to appear even in excess for some time. Hyperactivity of the thyrotropic and corticotropic hormones has also been noted in these first few postmenopausal years. No parallel phenomena have been recorded for the male. If the

TABLE 2

Daily intakes of cholesterol, fat and protein for men and women over 50 years of age

AGE GROUP	NO.	AV. BODY WEIGHT	FAT				CHOLESTEROL			PROTEIN		
			Total		per kg	% of calories	Total		per kg	Total		per kg
			mean	s.e.[1]			mean	s.e.[1]		mean	s.e.[1]	
years		*kg*	*gm*		*gm*		*mg*		*mg*	*gm*		*gm*
Men												
50–54	40	74.1	111	5	14.9	38	772	44	10	92	4	1.2
55–59	40	74.5	114	5	15.3	39	886	51	12	95	4	1.3
60–64	34	74.5	102	4	13.7	37	782	56	10	89	4	1.2
County Home	11	66.4	67	4	10.1	32	421	27	6	59	3	0.9
65–69	39	70.9	98	4	13.8	38	753	58	10	80	3	1.1
County Home	16	61.8	74	4	12.0	34	439	43	7	63	4	1.0
70–74	33	70.9	90	3	12.7	37	606	41	8	76	3	1.1
County Home	7	67.7	71	4	10.5	34	489	27	7	64	5	0.9
75–79	17	70.5	86	5	12.2	36	626	62	9	77	5	1.1
County Home	5	60.0	64	3	10.7	31	464	120	8	59	3	1.0
80 +	15	66.8	94	6	14.1	38	624	55	9	75	6	1.1
County Home	4	64.5	71	7	11.0	34	437	48	7	63	6	0.8
Women												
50–54	43	68.2	77	3	11.3	39	466	26	7	60	2	0.9
55–59	49	65.9	71	3	10.8	37	438	28	7	62	3	0.9
60–64	55	65.4	76	3	11.4	38	467	30	7	62	2	0.9
65–69	51	65.0	76	3	11.7	37	464	29	7	64	2	1.0
70–74	38	65.6	70	3	10.7	38	487	27	7	65	3	1.0
75–79	24	63.2	61	3	9.6	36	395	37	6	53	3	0.8
80 +	12	55.9	55	4	9.8	35	301	46	5	47	3	0.8
Totals												
Men	216	72.4	99	5	13.7	38	744	51	10	83	4	1.1
Women	272	65.0	72	3	11.1	37	449	29	7	59	3	0.9
County Home men	43	64.1	70	4	10.9	33	445	46	7	61	4	0.9

[1] Standard error.

level of circulating cholesterol is affected by sex hormone and adrenocortical hormone production a decrease in this level might be expected during the first 5-year period following the completion of the menopause. Such a drop occurred in the mean serum cholesterol level of these women in the age group 54 to 59 years. The rise which followed in the next age group was maintained up to 75 years. After 75 years in both men and women a sharp fall in the level occurred, but whether this fall can be related to steroid hormone production is not clear. However, if the drop in serum cholesterol level of the women between 54 and 59 years is ascribed to the utilization of cholesterol in ovarian and other steroid hormone production, the succeeding sustained high level in the 55 to 75-year age group may point to little variation in steroid hormone production in these 20 years.

The drop in serum cholesterol in women in the 54- to 59-year age group with the following sustained high level is not paralleled in the male. This may be due to the rather definite and similar menopausal age in women as contrasted with the irregular and highly variable if not controversial occurrence of climacteric in men.

Effect of age

The distribution of three groups of serum values among the subjects, segregated by sex and age by 5-year intervals and by decades, was examined. It was found that the men had serum cholesterol levels fairly evenly divided in the ranges, less than 220, 220 to 260, and more than 260 mg%. There were 37, 34 and 29% of the total number of men in each of these groups. Similarly the women had ranges of less than 240, 240 to 280 and more than 280 in 29, 35 and 36% respectively of the total group. These ranges were therefore chosen for attempted correlations with diet and physical factors.

The percentage of men in the group having the lowest serum levels, less than 220 mg%, increased steadily from 50 to over

70 years of age and that of those with the highest levels, more than 260, correspondingly decreased. This is shown in the 5-year means in table 1, both for the men living in their own homes and those in the county home. A similar comparison for the women yielded a different result. The percentage of those with the lowest serum concentrations was greatest in the 50- to 59-year group, lowest in the 60- to 69-year group and intermediate in the group over 70. The highest serum values were seen to predominate in the 60- to 69-year group. Thus there appeared to be an increase in cholesterol concentration in women in the 60- to 69-year interval as compared with both the younger and the older women. This is shown in table 1 in the drop in mean values between 55 and 60 years with a sustained rise from 60 to 74 and a drop thereafter. There was no variation in the proportion of free to total cholesterol in any group. This remained between 27 and 28% throughout.

The age trend in men agrees with the well supported values of Keys et al. ('50), of Kornerup ('50) and of Hobson, Jordon and Roseman ('53). All levels reported by the latter group however, are consistently 11 to 16% higher than those of the other investigators. The higher levels of the women are also in agreement with values reported by Kountz et al. ('45) and Hobson et al. ('53). The former group found a mean cholesterol level of 237 mg% in 94 institutionalized women from 41 to more than 81 years of age and the latter reported 310 for 141 women 61 to 87 years of age living in their own homes. These means are comparable with our finding of 270 mg% in 296 women 50 to more than 80 years old and living at home.

Effect of cholesterol intake

The daily intakes of cholesterol, fat and protein of the subjects in this study are shown in table 2. A positive correlation, $r = 0.11$ in men and 0.12 in women, was found between serum cholesterol levels and cholesterol intakes. This is significant at the 5% level. To illustrate this correlation the

cholesterol intakes of the men were divided into three groups, less than 450 mg% per day, 450 to 750, and more than 750. There were 24, 40 and 36% of all the men in these groups. Those of the women had to be divided into 4 groups in order to obtain a fair division, less than 300, 300 to 449, 450 to 600 and more than 600. There were 14, 36, 20 and 30% of the women in these 4 groups. When the percentage of men having

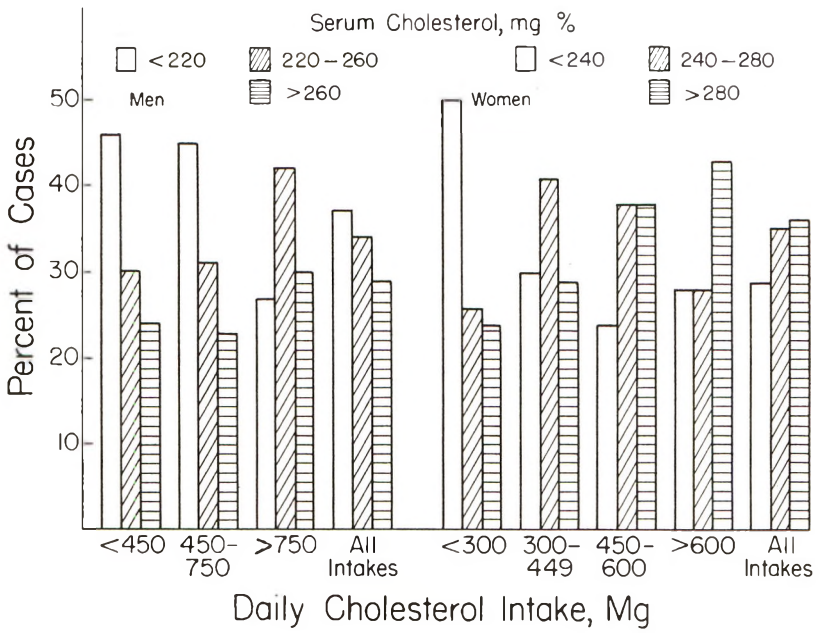

Fig. 2 Distribution of three ranges of serum cholesterol values in groups of men and women with varying cholesterol intakes.

the serum ranges previously described were arranged under the three groups of cholesterol intakes (fig. 2) it was seen that the largest percentage of those in the lowest intake range had the lowest serum concentrations and this decreased in the group having the highest intakes. The same relationship was evident when the values for the women were similarly arranged. When the converse grouping was made in both men and women a somewhat similar relationship emerged.

The groups having the lower serum cholesterol levels were found to contain more persons with low cholesterol intakes than the groups with higher serum levels, and of those with the highest serum levels a larger percentage had the highest cholesterol intakes. These differences were not as regular nor as striking as those seen in a previous study of serum ascorbic acid and intake of these same subjects (Morgan, Gil-

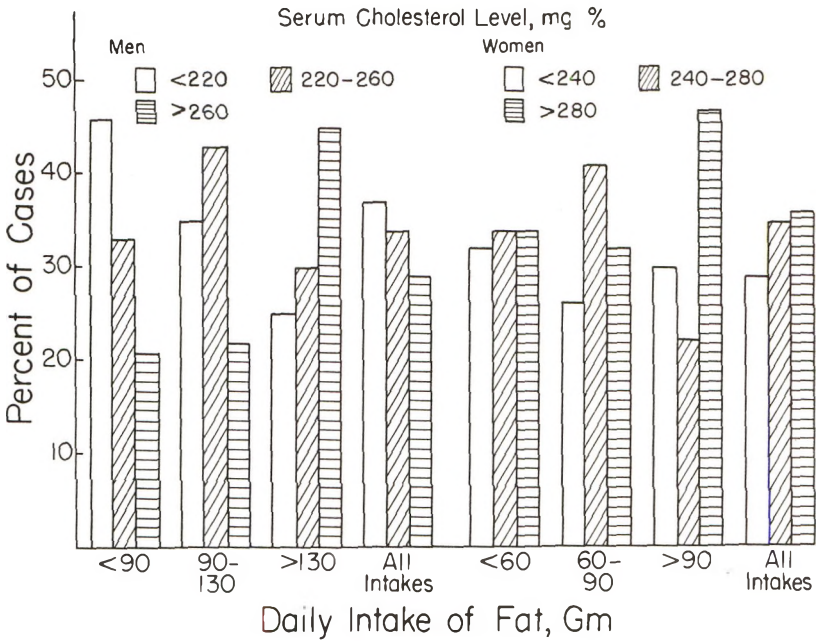

Fig. 3 Distribution of three ranges of serum cholesterol values in groups of men and women with varying fat intakes.

lum and Williams, '55). The trend however is unmistakable. This is in agreement with the conclusions of some observers and in disagreement with those of others. Morrison ('51), Messinger et al. ('50) and Moreton ('47) have given some evidence tending to support the hypothesis while Keys ('49, '50) and Wilkinson et al. ('50) maintain that cholesterol intake within reasonable limits does not affect the serum level.

Effect of fat intake

It has been vigorously asserted that serum cholesterol levels are positively correlated with total fat intake (Keys, '52; Gofman, '52a; Morrison, '51) and also that this correlation is positive only for fat of animal origin (Kinsell et al., '53). In this study a positive correlation, $r = 0.15$, was found between total dietary fat and serum cholesterol levels for the men. This is significant at the 1% level. The corresponding value for women was 0.09, barely significant at the 5% level.

TABLE 3

Relationship between dietary fat and dietary protein and cholesterol

DAILY FAT INTAKES	PER CENT OF CASES WITH THESE PROTEIN INTAKES IN GRAMS			PER CENT OF CASES WITH THESE CHOLESTEROL INTAKES IN MILLIGRAMS			
gm							
Men	< 70	70–90	> 90	< 450	450–750	> 750	
< 90	53	42	5	37	48	15	
90–130	9	42	49	4	43	53	
> 130	0	7	93	0	14	86	
Women	< 50	50–70	> 70	< 300	300–449	449–600	> 600
< 60	56	½	2	32	49	12	7
60–90	17	60	23	8	36	25	30
> 90	0	25	75	0	16	19	64

The fat intakes were divided into three approximately equal groups, less than 90, 90 to 130, and more than 130 gm per day for the men; for the women, less than 60, 60 to 90 and more than 90. Again the subjects with the three ranges of serum cholesterol previously described were grouped in these intake ranges as shown in figure 3. There appears to be an orderly relationship between the proportions of high serum cholesterol concentrations and high fat intakes and of low concentrations with low intakes in both men and women. The relationship is more regular in the men than in the women. The converse grouping also showed the same relationship, the largest percentage of both men and women having the lowest range of fat intakes was found in the

groups with the lowest serum levels and the highest fat intakes occurred correspondingly more frequently in the subjects with the highest serum cholesterol levels.

The intake of fat of animal origin was calculated and the same comparisons made with serum cholesterol concentrations as had been done with total fat intakes. No direct proportionality between these values for the men can be seen and only a dim relationship for the women. The converse diagrams followed the same pattern. The serum cholesterol levels were definitely more proportional to the total than to the animal fat intakes. The close correlation between fat and cholesterol contents of the diets is shown in table 3.

Effect of protein intake

Since lipotropic agents have been thought to have some value in reduction of serum cholesterol levels (Davidson, '51) a comparison of these levels was made with the protein intakes of the subjects. The correlation statistic r was $+0.12$ for the men and $+0.06$ for the women. The correlation for the men is significant at the 5% level. This is surprising since a negative correlation might have been expected if the methionine content of the protein had exerted the reputed lipotropic effect. In order further to test this supposition, the dietary protein of animal origin was calculated since this type of protein generally provides more methionine than does vegetable protein. The usual comparison of three ranges of serum cholesterol was then made with three ranges of total protein intake which were found to divide the groups into fairly even numbers. When the groups with a given range of protein intake were divided into the percentages having the three ranges of serum cholesterol it was evident that there was a slight positive relationship in both men and women between the low levels of intake and low serum cholesterol (fig. 4). When a similar comparison was made between daily intakes of protein of animal origin and serum cholesterol levels a similar but less convincing relationship emerged. The con-

verse arrangement, division of the groups within a given range of serum levels into the percentages of those with a high, intermediate or low range of total or animal protein intake likewise presented a slight positive correlation. There was no suggestion of a depressing effect by dietary protein intake upon the serum cholesterol levels. The obvious and close

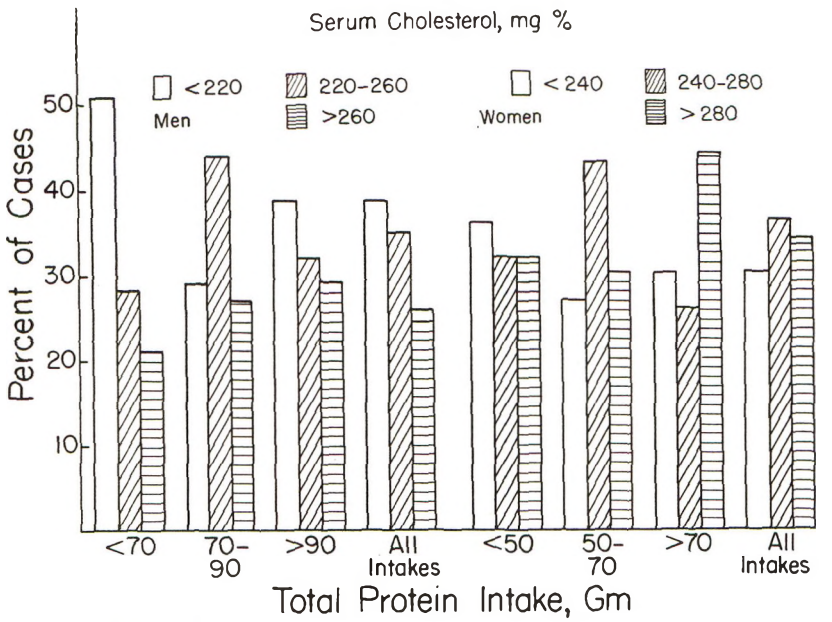

Fig. 4 Distribution of three ranges of serum cholesterol values in groups of men and women with varying protein intakes.

correlation between fat content and both the protein and cholesterol contents of the diets is seen in table 3. The influence of the dietary protein may therefore be chiefly a reflection of the concomitant fat content.

Possible relationship of serum cholesterol to serum ascorbic acid levels

Some relationship between ascorbic acid and cholesterol metabolism has been suspected, chiefly because of the role

that both of these substances seem to play in steroid hormone secretion (Long, '47). Since both of these serum constituents were found in significantly higher concentration in women than in men (Morgan, Gillum and Williams, '55) a comparison of ranges was made. Each of the groups of men with the three usual levels of serum cholesterol were separated into three groups with the varying serum ascorbic acid levels

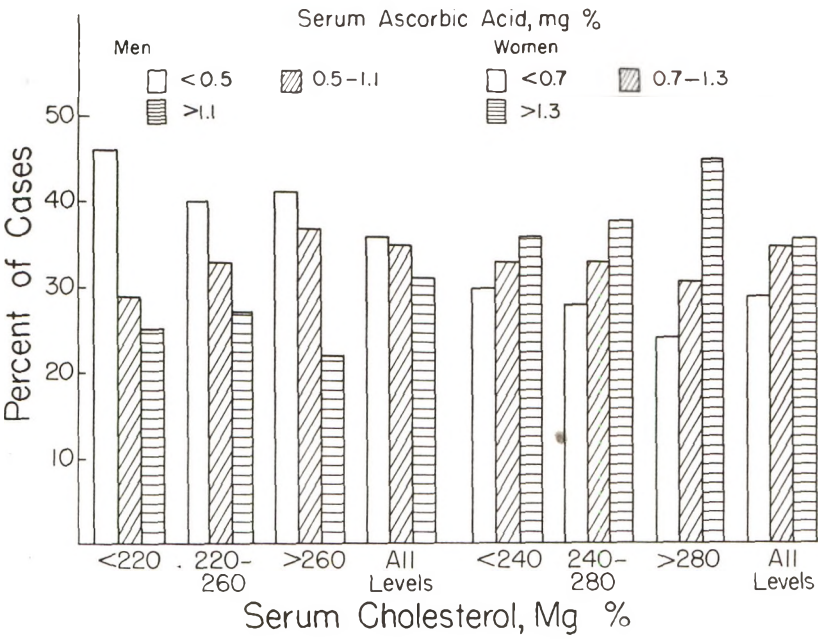

Fig. 5 Relationship of serum ascorbic acid and cholesterol levels.

used. No significant relationship appeared. The values for the women with the three usual cholesterol levels were also divided according to serum ascorbic acid levels. Here a slight positive relationship emerged (fig. 5). When the converse arrangement was made, a similar slight correlation appeared for the women but not for the men. The age-serum level curves of the women exhibited a peak in the 60- to 64-year-old group for both ascorbic acid and cholesterol followed by a fall in the former but not in the latter. These curves are not parallel

in the values of the men, although a similar peak in serum ascorbic acid occurred in the 60- to 64-year-old group. The serum cholesterol values of the men declined rather evenly up to 75 years.

The parallel peak in the serum ascorbic acid level of women in the age group 60 to 64 years is an interesting coincidence if nothing more. Much attention has been given to the supposed simultaneous depletion of ascorbic acid and cholesterol following stimulation of the adrenal cortex by corticotropin but since there is no certainty as to the mechanism of steroid hormone manufacture an acceptable explanation for the phenomena is not available. Certainly the difference in level of these two serum constituents in the sexes and with advancing age have striking similarities.

Body weight

Overweight in the subjects examined has been related to the maintenance of hypercholesteremia and the occurrence of coronary disease (Master et al., '53) (Gofman, '52b). To test this hypothesis the body weights of these subjects were compared with their serum cholesterol levels. The under or overweight values were obtained by comparison with Metropolitan Life Insurance tables (Metropolitan Life Insurance Co., '42, '43) for weight and height, the excess weight being calculated from the normal weight at 25 years of age for men and women of medium body build. All the subjects, men and women separately, were divided into 5 groups, those more than 20% underweight, 10 to 20% underweight, 10% under to 10% overweight, the so-called normal weight group, 10 to 20% overweight and more than 20% overweight. The proportions of men and women in these groups and the percentage in each of the three ranges of serum cholesterol previously described are shown in figure 6. More women than men were underweight, 29% against 21%, and also overweight, 24% against 22%. Fifty-eight per cent of the men and 48% of the women were in the normal weight group. In the men there

appears a small positive correlation of extreme underweight and extreme overweight with serum cholesterol but in the women there was no correlation or a slight negative one. This may also be due to the character of the diet (fig. 7). The overweight men had greater fat and cholesterol intakes than the

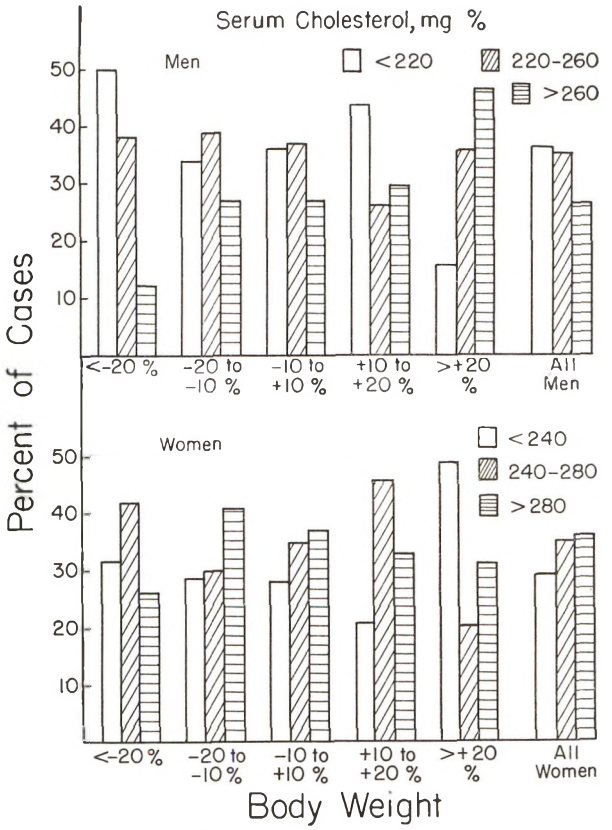

Fig. 6 Relationship of serum cholesterol levels to variations in body weight.

normal or underweight men. No such difference was seen in the intakes of the overweight women. A similar sex difference in regard to the relationship between coronary disease and hypertension and obesity was noted by Master, Jaffe and Chesky ('53). Hobson et al. ('53) found no correlation be-

tween obesity and serum cholesterol in either men or women. Walker ('53), in men and women 30 to 60 years old, found a direct correlation between overweight and serum cholesterol, more marked in the S_f12–20 lipoprotein fraction than in the total cholesterol and more pronounced in men than women.

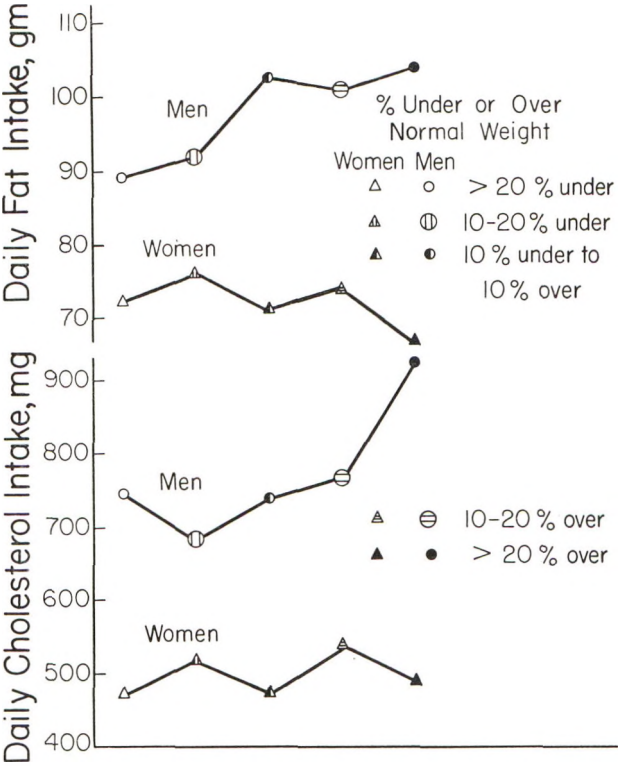

Fig. 7 Relationship of underweight and overweight to fat and cholesterol intakes.

Blood pressure

The blood pressure, diastolic and systolic, of these subjects was compared with the serum cholesterol levels without significant result. The distribution of subjects with diastolic pressures of less than 90, 90 to 100 and more than 100 among the three ranges of serum cholesterol levels was not signifi-

cantly different from the distribution of the total serum levels. The systolic pressures used were less than 140, 140 to 180 and more than 180. In both men and women again no significant correlation with serum levels emerged. This confirms the findings of Hobson et al. ('53).

The men in the county home

The serum cholesterol levels of the 43 men over 60 years of age living in the county home were significantly lower than those of the 234 men living in their own homes. This same lower value had been seen in the serum ascorbic acid levels of these men (Morgan, Gillum and Williams, '55). The average body weight of the men in the county home was less than that of the other men and even slightly less than that of the women. Their calorie intake per kilogram per day, 29, was midway between those of the other men, 33, and the women, 26, and their protein intake per kilogram per day, 0.9 gm, was the same as that of the women and less than that of the other men, 1.1. Their fat and cholesterol intakes were likewise lower than those of the other men, 70 gm and 445 mg daily and nearly identical with the average fat and cholesterol intakes of the women.

If it is assumed that the lower intakes of fat and cholesterol are responsible for the low serum cholesterol levels of these 43 men, then the high serum values of the women who had nearly the same intakes must be ascribed to other, perhaps hormonal, causes.

There is some evidence that men in institutions, in poor economic circumstances and in less than normal health have relatively low serum cholesterol concentration. Schaefer et al. ('53) noted the significantly lower mean cholesterol levels of their sick and economically underprivileged male subjects in the older age groups as compared with Keys' averages for healthy men in relatively comfortable economic conditions. The values quoted by Kountz et al. ('45) for a somewhat similar hospital population are likewise low compared with

both our findings and those of Keys. Thus their 94 men between 51 and 80 years of age had a mean cholesterol level of 199 mg% compared with 241 for our 234 men living at home and 209 for our 43 men in the county home. The low serum cholesterol values reported by Keys ('53) for clinically healthy but poor Spaniards and Neapolitans may be cited also in this connection.

The physiology of stress may be illustrated in the blood characteristics of the men in the county home. Except for a few diabetics the blood sugar levels were low, serum ascorbic acid and cholesterol and volume of packed cells were significantly lower than those of the other men. Both serum levels and intakes of vitamin A and carotene of the men in the county home were also lower than were those of the men living in their own homes.

SUMMARY

The serum cholesterol levels of 530 supposedly normal persons, 234 men and 296 women, 50 to more than 80 years of age living in their own homes were found to vary from 106 to 720 mg per 100 ml blood, the means for the men being 241 ± 8 and for the women, 270 ± 8. The free cholesterol was 27 to 28% of the total and this proportion did not vary significantly in any group. The mean level for 43 additional men over 60 years of age, living in the county home, was 209 ± 12.

The women had significantly higher levels than the men between the ages of 60 and 80 years. In both sexes a sharp drop in the levels occurred at 75 or 80 years. There was a downward trend in the levels of the men with each decade but a sustained high level was shown by the women in the groups from 60 to 75 years of age after a sharp drop in the 54- to 59-year group. This drop may be due to a post-menopausal spurt in steroid hormone production.

A positive correlation of the order of 0.12 was found between dietary cholesterol and serum cholesterol levels in both men and women and a similar correlation, 0.15 in men, 0.09

in women, between fat intake and serum cholesterol levels. When intake of fat of animal origin only was considered the correlation was lower.

A slight positive correlation with protein intake was also found and this was thought to stem from the similar dietary occurrence of fat, cholesterol and protein.

A striking parallelism was noted between the serum ascorbic acid and serum cholesterol levels in women but not in men. A possible relationship between these blood constituents and steroid hormone manufacture and circulation is suggested.

Extreme under- or overweight, 20% or more, in men but not in women, was found to be associated with low and high serum cholesterol levels respectively as well as with low and high intakes of fat and cholesterol. No such relationship was seen in the groups with smaller deviations from the normal weight.

No relationship between blood pressure and serum cholesterol levels was found in any of the groups.

It is clear that standards for hypercholesteremia must be different for men and women and that dietary fat and cholesterol are associated positively with the serum cholesterol levels.

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EFFECTS OF FASTING ON BLOOD NON-PROTEIN AMINO ACIDS IN HUMANS[1]

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Previous reports (Charkey et al., '53, '54) have indicated that in chicks the blood levels of free amino acids are affected by withdrawal and by replacement of feed; that the several amino acids thus studied are not all affected alike; and that lysine occupies in this respect a somewhat unique position in that it shows a marked rise during fasting.

It was suggested that the increase in the level of lysine in blood might be explained by assuming that lysine is not susceptible to deamination by chicks. Such an assumption would appear to be plausible on the basis of the established lack of amination of lysine precursors (Schoenheimer, '49; Jackson and Chandler, '39; Foster et al., '38, '39; Weissman and Schoenheimer, '41; Clark and Rittenberg, '51; Berg, '36; and Bauer and Berg, '43). More recently it has been shown (West and Carter, '38; Elliott and Neuberger, '49, '50) that threonine is less readily formed by precursor amination than other amino acids with the exception of lysine. When threonine was later found (Charkey et al., '54) to display in chicks a fasting rise similar to that of lysine, the correlation between (a) lack of precursor susceptibility to amination and (b) blood rise during fasting could not fail to arouse interest. This interest was not lessened by the fact that this peculiar property of lysine and threonine was found to be a function of the age of the chicks used.

[1] Scientific Series Paper no. 432, Colorado Agricultural Experiment Station.

The foregoing, plus the fact that this behavior of lysine was not seen in rats (Wiss, '48a, '48b, '49; Henderson et al., '49), indicated the need for further studies of animals of different species and degrees of maturity. Rats and chicks appear to be the only species so far subjected to fasting studies of this type. Accordingly the present study of adult humans was undertaken, with the deliberate purpose of using as subjects members of an entirely different species than used previously, at an entirely different degree of maturity.

EXPERIMENTAL

Six adult human volunteers (5 men, one woman) in good health, ranging in age from 22 to 42 years, served as experimental subjects. Blood levels and urinary excretion of specific amino acids before, during and after a 48-hour fast constituted the principal experimental measurements made. The fasting and sampling schedule was as follows:

8 A.M., 1st day	Begin collection of FF [2] 24-hour urine specimen
8 A.M., 2nd day	Withdraw FF 10 ml blood specimen
	End collection of FF urine specimen
	Begin 48-hour fast
8 A.M., 3rd day	Begin collection of OF [2] 24-hour urine specimen
8 A.M., 4th day	Withdraw OF 10 ml blood specimen
	End collection of OF urine specimen
	Begin collection of RTF [2] 24-hour urine specimen
	Terminate fast
3 P.M., 4th day	Withdraw RTF 10 ml blood specimen
8 A.M., 5th day	End collection of RTF urine specimen

Thus the FF urine specimen represented the 24-hour period just prior to the fast, the OF urine specimen represented the second (last) 24-hour period of the 48-hour fast, the RTF urine represented the first 24 hours after conclusion of the fast. The blood samples were taken just prior to initiation of the fast (FF), just prior to its termination (OF), and about 6 hours after termination (RTF). Protein-free filtrates were prepared from all blood specimens immediately after collection, by the procedure of Folin ('30) for unlaked blood.

[2] FF — "full food," or pre-fasting conditions, OF — "off-food," or fasting conditions; RTF — "returned to food."

The protein-free filtrates were analyzed for total nitrogen by micro-Kjeldahl digestion and Nesslerization, and for tryptophan by the use of *Streptococcus faecalis* A.T.C.C. 9790 in a modification of the medium of Stokes et al. ('45) for amino acid assays. The remainders of the filtrates were made 1 N with concentrated HCl, autoclaved at 18 pounds pressure for 4 hours, neutralized with NaOH and filtered. The resulting hydrolysates were then assayed for lysine,[3] threonine, methionine, arginine, leucine and valine, using *S. faecalis* in the modified Stokes medium.

After measurement of volume, each urine specimen was divided into two portions, one of which was subjected to the hydrolysis procedure of Harvey and Horwitt ('49). This portion was assayed for lysine, threonine, methionine, arginine, leucine and valine. The untreated portion was used for measurements of tryptophan, specific gravity, total solids, pH, titratable acidity, total nitrogen, ammonia nitrogen, urea nitrogen, creatinine, and uric acid. All microbiological assays were performed by the use of *S. faecalis* in modified Stokes medium. Total nitrogen was determined by micro-Kjeldahl digestion and Nesslerization, ammonia nitrogen by zeolite adsorption and Nesslerization of the eluates. Creatinine was determined by the Jaffe reaction and colorimetry; and uric acid colorimetrically, according to the arsenophosphotungstic acid procedure of Benedict and Franke ('22).

RESULTS

The determined amino acid and non-protein-nitrogen values for blood are shown in detail in table 1. The urine amino acid contents are shown in comparable fashion in table 2. In table 3 are averages (only) for the entire group of subjects of the several incidental observations in urine.

[3] One run for lysine was made on the un-hydrolyzed protein-free filtrates. It was in part because of the low values in this assay that the hydrolysis was decided on. The values in subsequent lysine assays were much higher. Amino acids are known to occur in urine and in blood partly in the form of peptides. Scarcity of material precluded any study of this aspect for more than one amino acid.

TABLE 1

Amino acids and non-protein nitrogen of blood

SAMPLING STATE	SUBJECT [1]	BODY WT.	LYSINE	THREONINE	METHIONINE	ARGININE	LEUCINE	TRYPTOPHAN	VALINE	*NPN*
		kg	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
Full food	1	88.8	3.205	0.925	0.00	1.28	3.06	0.947	3.71	30.75
	2	59.2	4.66	2.27	0.260	2.00	4.12	0.712	5.05	32.0
	3	64.8	4.34	1.60	0.044	1.84	4.265	1.08	5.285	36.5
	4	67.5	8.11	4.68	1.00	4.10	. . .	0.928	11.4	52.25
	5	81.7	3.22	0.737	0.092	1.26	3.56	0.922	4.83	38.0
	6	74.8	4.34	2.195	0.079	2.25	4.375	0.927	4.615	36.5
	Av.	72.8	4.65	2.07	0.246	2.12	3.88	0.919	5.81	37.7
Fasting	1	86.0	2.72	0.610	0.00	0.895	5.16	0.663	6.46	21.8
	2	57.0	3.60	1.39	0.210	1.33	4.91	0.718	5.71	33.25
	3	62.5	3.495	1.23	0.195	1.375	7.12	0.557	8.65	32.0
	4	64.5	4.19	1.825	0.395	1.72	6.78	0.590	7.97	43.75
	5	78.4	3.965	1.47	0.280	1.625	6.715	0.678	8.825	38.0
	6	73.0	6.00	1.365	0.320	3.62	5.50	0.693	8.11	39.5
	Av.	70.2	3.995	1.315	0.233	1.76	6.03	0.650	7.62	34.7
Returned to food	1	87.5	2.31	0.480	. . .	0.815	. . .	0.810	. . .	32.3
	2	58.2	5.46	2.78	0.435	2.215	5.87	0.807	6.50	34.2
	3	63.2	5.19	1.85	0.465	1.75	5.565	0.923	6.955	42.25
	4	65.5	2.48	0.438	0.130	0.815	2.985	0.862	4.575	38.0
	5	79.5	6.03	3.06	0.545	2.265	6.185	0.863	6.775	57.5
	6	74.0	2.53	0.338	. . . .	0.691	2.84	0.933	3.36	26.0
	Av.	71.3	4.00	1.49	0.394	1.43	4.69	0.866	5.63	38.4

[1] Arranged in order of increasing age: Nos. 1 and 2, 22 years; No. 3 (woman), 26 years; No. 4, 27 years; No. 5, 37 years; No. 6, 42 years. All figures shown are averages of at least 4 measurements taken from not less than two "runs."

Examination of the results in table 1 makes it clear that lysine and threonine were by no means unique with respect to the blood level response to fasting. The lysine and threonine levels were reduced in common with total non-protein nitrogen and with three other amino acids, namely methionine, arginine, and tryptophan. Leucine and valine, on the other hand, rose appreciably during fasting. Thus in the present species and under these conditions, two entirely different amino acids displayed a type of response shown consistently in young chicks by lysine alone.

One of several reasons for carrying out a study in humans was that this permitted collection of 24-hour urine specimens. A possible serious weakness of studies based on blood analyses alone is that the question remains unanswered as to whether the blood values are established as a result of metabolic activity or by adjustments of excretory (renal) function to experimental treatments. Urinary excretion values permit at least an intelligent guess as to the true state of affairs.

The results in table 2 indicate that changes in urinary excretion from FF to OF, and from OF to RTF generally parallelled those in blood levels. From this it can be argued that blood levels governed excretion, and not the reverse. If the amount of excretion had been the primary regulator of blood levels, the urinary and blood values should have been inversely related.

The only instances in which urinary excretion did not parallel blood level change during fasting were those in which increases in blood levels occurred during fasting (leucine and valine). It might be argued that in these instances, the increases in blood levels resulted from reduction of renal excretion. But if so, it would be extremely difficult to explain the fact that none of the 5 fasting *reductions* in blood level was associated with *increased* excretion. It seems more plausible to suppose that the two observed fasting increases in the blood were true reflections of metabolic activity, that they did not occur until late in the fasting period, and that

TABLE 2

Urinary excretion of amino acids

SAMPLING STATE	SUBJECT [1]	LYSINE	THREONINE	METHIONINE	ARGININE	LEUCINE	TRYPTOPHAN	VALINE
		mg/24 hrs.	*mg/24 hrs.*	*mg/24 hrs.*	*mg/24 hrs.*	*mg/24 hrs.*	*mg/24 hrs.*	*mg/24 hrs.*
Full food	1	96.35	74.9	87.3	36.5	34.55	38.9	45.9
	2	64.9	44.4	59.45	20.1	24.2	24.9	29.5
	3	89.2	51.75	81.95	29.8	26.6	19.25	32.65
	4	87.3	39.65	86.9	36.15	35.0	24.1	42.95
	5	138.5	60.5	84.2	30.65	30.9	39.9	40.25
	6	91.7	71.85	79.35	37.1	39.35	35.4	46.45
	Av.	94.7	57.2	79.9	31.7	31.8	28.7	39.6
Fasting	1	52.45	40.2	61.6	26.4	31.75	25.0	36.1
	2	61.9	21.9	51.0	18.85	22.45	25.5	32.4
	3	56.75	29.7	58.1	18.3	23.1	13.0	29.2
	4	44.75	26.4	59.2	20.6	26.55	16.15	32.9
	5	89.95	39.4	69.6	20.55	31.1	25.9	40.55
	6	70.5	40.8	69.3	24.9	37.2	31.6	41.75
	Av.	62.7	33.1	61.5	21.6	28.7	22.9	35.5
Returned to food	1	89.5	67.55	72.9	28.6	35.45	36.9	44.1
	2	57.6	37.6	64.3	32.15	28.9	34.2	38.6
	3	81.25	46.35	60.3	22.3	32.6	14.85	43.0
	4	73.75	38.3	59.0	24.2	33.0	26.15	42.0
	5	107.	59.25	65.9	27.9	34.6	37.65	41.5
	6	82.5	64.6	85.6	35.6	41.75	34.6	50.8
	Av.	81.9	52.3	68.0	28.5	34.4	30.7	43.3

[1] Arranged in order of increasing age: Nos. 1 and 2, 22 years; No. 3 (woman), 26 years; No. 4, 27 years; No. 5, 37 years; No. 6, 42 years. All figures shown are averages of at least 4 measurements taken from not less than two "runs."

this accounts for a lack of *apparent* parallel increase in excretion, inasmuch as the urine specimens were 24-hour collections. Only further investigations, with more frequent sampling of urine, or of both blood and urine, can provide a reliable answer to this question.

The continued drop (OF to RTF) in blood arginine, and the rise in blood methionine were not equivalently reflected in urinary output. Here again it is not possible to assess clearly the importance of renal function as a regulator of

TABLE 3

Incidental urine observations[1]

	FULL FOOD (FF)	FASTING Off food (OF)	FULL FOOD Returned to food (RTF)
Body weight (kg)	72.8	70.2	71.3
Urine volume (ml/day)	1133	951	975
Specific gravity	1.0275	1.025	1.029
pH	5.91	5.30	5.42
Titr. acidity (ml 0.1 N acid/day)	338	416	393
Total N (gm/day)	16.2	14.2	23.3
Urea N (gm/day)	11.2	10.7	14.8
Ammonia N (gm/day)	0.442	0.519	0.872
Uric acid N (gm/day)	0.324	0.115	0.304
Creatinine N (gm/day)	0.724	0.646	0.699

[1] Figures shown are averages of data for all 6 subjects.

blood levels. In these cases the blood specimens were taken only 6 hours after return to food. In the next 18 hours, during all of which the RTF urine specimen was still being collected, the blood picture probably changed materially. Again the cases noted constitute the exceptions to the usual response. Generally speaking the agreement between changes in blood levels and changes in urinary outputs is quite close, considering the stated conditions.

The data shown in table 3 were collected with the idea of furnishing a broad base for the interpretation of the data on amino acids, and of exposing any gross variation of any individual subject from "normal," should such occur. Since

nothing beyond reasonable and customary variation from average was encountered, only the average values are given. They represent a picture of urinary discharge such as might be expected from fasting. There was, during fasting, a reduction in most urinary components; and acidosis, reduced after return to food. Ammonia excretion increased during the fast, however, and was still higher during the first 24 hours after conclusion of the fast. This probably is explainable on the basis of use of ammonia for excretion of acid; inasmuch as the acidosis still was present for at least part of the first 24 hours after return to food. Ammonia formation as a response to acidosis is generally accepted.

There is, then, nothing in the present studies constituting a serious objection to the interpretation of changes in blood free amino acid levels as reflections of intermediary metabolic activity.

DISCUSSION

The original purpose of this experiment was simply to test the possibility that in a different species (human) the response to fasting, expressed in terms of which amino acid blood levels are increased and which are decreased, might be similar to that observed previously in chicks. That such is not the case has been amply demonstrated by the results.

These same results invite speculation on a hypothesis of probably considerable fundamental importance, namely that L-amino acids metabolically unavailable by amination of structural analogues will rise in blood level during fasting, and the converse. The remainder of this discussion is a development and evaluation of such a hypothesis.

A correspondence has been noted between(a) amino acids which increase in the blood of chicks during fasting, and (b) amino acids which are metabolically unavailable by amination of structural analogues. As a working hypothesis to account for the observed relationship, it has been assumed that an amino acid difficult to obtain by precursor amination

in tissues can be eliminated only slowly by deamination in tissues. This could readily account for an increased blood level during short term fasts. Conversely then, an amino acid found to increase in blood level during fasting may do so because the metabolic machinery has difficulty in attaching amino groups to the structural analogues, in converting them to the amino acids. Such a postulation is nearly as plausible as, and is based on the accepted theory that the lack of a necessary enzyme hinders the reaction catalyzed by it from proceeding in either direction.

Utilizable D-amino acids are enzymatically converted to L-amino acids via the corresponding keto acids by deamination of the former, followed by amination of the latter to form the L-compounds. Hence information regarding metabolic utilization of D-amino acids can be applied in testing the validity of the hypothesis being presented.

In light of the foregoing, it would be predicated from the present data that the D-isomers of lysine, threonine, methionine, arginine and tryptophan are readily utilizable in human metabolism; and that the D-isomers of leucine and valine are *not* readily utilizable by humans.

Examination of the literature revealed that D-leucine is indeed *not* able to replace the natural isomer in adult human nutrition (Rose, '49). Data on D-valine in human nutrition are not available. Thus for the two amino acids showing a fasting rise in the present study, there is no evidence contradictory to the idea that a fasting rise is correlated with precursor unavailability; and in one case there is direct confirmation.

Of the 5 amino acids showing fasting reductions in blood level, Albanese ('47) has listed 4 as being available in human metabolism from the D-isomers. Only tryptophan was listed as metabolically unavailable from the D-isomer. This was a qualified listing, inasmuch as the author pointed out in the same article that acetyl-D-tryptophan is metabolically available to humans; and in a general summary of utilization of D-amino acids in several species stated, " . . . it appears that

the adult human is able to utilize the unnatural forms to a greater degree than either the rat or the mouse. Apparently mechanisms for the optical conversions of the unnatural amino acids exist in man which differ qualitatively and quantitatively from those prevailing in other mammals. The findings with acetyl-DL-tryptophan suggest that this conversion may be effected through a biological acetylation and deacetylation mechanism which is more highly developed in man than in the lower mammals.'' Thus even tryptophan cannot be definitely stated to be unavailable to humans in the D-form.

Accordingly, the results of this study establish a high degree of correlation, within the group of amino acids studied, between the fasting rise in blood non-protein amino acid level and precursor unavailability by metabolic amination in the human species. Since a similar conclusion was reached in earlier studies with chicks, the authors are encouraged to suggest that the phenomenon may be general, in this wise: *In a given species at a given degree of maturity, an amino acid which is not metabolically derivable from the* D-*isomer or other structural analogues is increased in blood level as a result of short-term, total fast.*

SUMMARY

1. Fasting in adult humans led in 48 hours to increased blood levels of leucine and valine. Blood levels of 5 other amino acids, namely lysine, threonine, methionine, arginine and tryptophan were simultaneously *reduced* by fasting.

2. The response of adult humans to fasting, in terms of blood non-protein amino acid levels, was entirely different from that found in chicks up to 6 weeks of age, in that the blood levels of different amino acids were increased as a result of fasting.

3. Examination of the literature revealed a correspondence, for the human species, between amino acids exhibiting a blood level rise during fasting and those not metabolically available by tissue conversion of structural analogues. A similar relationship had been found earlier in chicks. Hence

it is suggested that a correspondence, between amino acids exhibiting increased blood levels due to fasting and those metabolically unavailable by precursor amination, may hold true for a variety of species.

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THE EFFECT OF HIGH LEVELS OF TERRAMYCIN OR STREPTOMYCIN ON GROWTH, REPRODUCTION AND LACTATION OF THE RAT [1]

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ONE FIGURE

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Mickelson ('53), Jukes and Williams ('53) and Stokstad ('54) have reviewed various aspects of antibiotics in nutrition. In general, numerous beneficial results have been attributed to these substances, ranging from vitamin-sparing actions (Linkswiler et al., '51; Lih and Baumann, '51; Daft and Schwarz, '52; and Sauberlich, '52) to effects on the nutritive value of various proteins (Pecora, '52). Most of these investigations were of short duration, i.e., less than 16 weeks.

French et al. ('54) in life-span studies showed that terramycin and streptomycin at a level of 0.04% in the diet decreased the life expectancy of albino rats. Theiss ('51) reported that penicillin inhibited lactation in 5 human subjects being treated for infections. Perin et al. ('50) reported a possible abortifacient action of penicillin in humans and mice. Because of these results and because of the increased use of these drugs in nutrition and as therapeutic agents, it was thought advisable to investigate further the effects of long-term feeding of these antibiotics. The need for such work has been commented on by Heilman and Rake ('49).

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The value of reproduction and lactation measurements as criteria for assessing the nutritive value of a ration has long been recognized. French et al. ('52) used such a technique for the comparison of three complete diets. Stern and McGinnis ('50) investigated the effects of aureomycin or streptomycin on lactation in the rat. Their investigation, however, did not include effects on reproduction as the experimental diets were fed only after parturition. It was our objective to investigate the effects of long-term feeding on reproduction as well as on lactation. During the course of the experiment, it was also possible to investigate post-weaning growth, the amount of active antibiotic excreted in the feces, and the effect of these drugs on the fat content of the liver.

EXPERIMENTAL PROCEDURE

Diets. Three diets were used: a control diet, a terramycin-supplemented diet, and a streptomycin-supplemented diet. The basal diet mixture was finely ground Rockland Farms complete rat diet. The antibiotic-supplemented diets consisted of the basal diet plus 0.04% of either terramycin hydrochloride or streptomycin sulfate (in terms of the pure bases, this is 0.036% and 0.030%, respectively). These amounts were chosen by French et al. ('54) to furnish several times the concentration commonly used for growth stimulation in an effort to control respiratory disease, and were observed to decrease the life span of albino rats. The basal diet supplied all nutrients as set forth in the optimal allowance recommendations of Griffith and Farris ('42) and Russell ('48) for reproduction and lactation in rats. The diets were ground and mixed at 6-week intervals. Food was given to the animals on an ad libitum basis and food consumption was recorded weekly. The diets were kept under refrigeration until used. The environmental temperature was maintained at 27 $\pm$ 2°C., with few exceptions.

Selection and disposition of the rats. Thirty female and 10 male Wistar strain rats were placed on each diet at weaning age (average 23 days). A minimum weight of about

45 gm for the females and 50 gm for the males was used as a criterion for starting healthy young animals on experiment. An attempt was made to use one of three littermates on each of the diets. Where this was not possible, the placement of the rats on the diets was adjusted so as to have the same degree of pairing between any two of the diets. Distribution according to weight between the diets, within the limits of the first criterion of pairing, was accomplished so as to have approximately equal average starting weights of rats on each diet.

Reproduction and lactation. The breeding techniques used were those of French et al. ('52) and the same data were recorded. Sibling matings were avoided. The animals were 120 days old when first bred, and the females had all attained a minimum weight of 230 gm. There was no cross breeding between diets. At time of parturition, the dam was weighed, and the young counted and weighed, and weighed again on the third day post partum. At this time the litter was reduced to 6 young, keeping the three heaviest of each sex. The dam and each reduced sex group were again weighed on the 14th and 21st days after parturition. The dam was considered sterile if there was no indication of gestation after three successive matings to different proven males. All females were bred a second time one month after the weaning of their first litters. During this second reproduction period, the same procedure was followed and the same data recorded. In addition, the food consumption of the dam was measured from the third to the 14th day after parturition. The collection of data on lactation was limited to those dams whose litters could be reduced to 6 young.

A smaller group of weanling rats consisting of one male and one female from each litter of the second reproduction period of the first generation, was used to determine second generation effects of the antibiotics on reproduction and lactation. This second generation group was limited to 16, 18, and 14 females on the terramycin, streptomycin, and

control diets, respectively. In no instance were any changes made in their diets.

Excretion of antibiotics. Four-day collections of feces were made three times on groups of 4 littermate females on each diet. This gave an accumulation of feces for 48 rat days on each of the three diets. Feces were preserved with chloroform or drying or both. Terramycin in the preserved feces was assayed according to Kersey's procedure ('50). Streptomycin was assayed by the Food and Drug Administration method using *B. subtilis* in the plate technique as outlined in the Federal Register ('53).

Fat content of livers. Livers were obtained from the 10 first generation males on each diet and from 15 littermate females on each diet one month after the weaning of their second litters. The livers were frozen, dried in a vacuum desiccator over sulfuric acid, ground and extracted with anhydrous ethyl ether in a Soxhlet apparatus.

RESULTS AND DISCUSSION

Growth. Records were kept of the food consumption and weight gains of the rats (10 males and 30 females on each diet) from the time they were weaned and placed on experiment until such time as they were bred (120 days of age). These data are presented graphically in figure 1. It is apparent that the antibiotics exerted no significant effect on the growth rate of the females. There was some tendency toward increased size in the males, but the number of these animals was too small to warrant statistical analysis.

Reproduction. A good overall index of reproductive performance is the birth of large litters of living young of sufficient birth weight to insure survival. Other measures of reproductive performance used were: (1) the ability to conceive, (2) the number of young born per litter, (3) the birth weight of the young, (4) the number of young surviving to the third day, and (5) the percentage of animals bred that produced at least one live young. Results (table 1) showed no significant difference between diet groups in size of litters,

birth weight of young, or average number of young per litter surviving to the third day. The percentage of the dams bred that produced at least one live young dropped from first reproduction period highs of 97, 93, and 93% for terramycin, streptomycin, and control, respectively, to 77, 47, and 57%, respectively, for the second period. This was significant ($p < 0.02$) on comparison of the results for the streptomycin and terramycin groups. At three days post partum, the percentage of litters with one or more viable young was further reduced in the streptomycin group to 43%. The reproductive

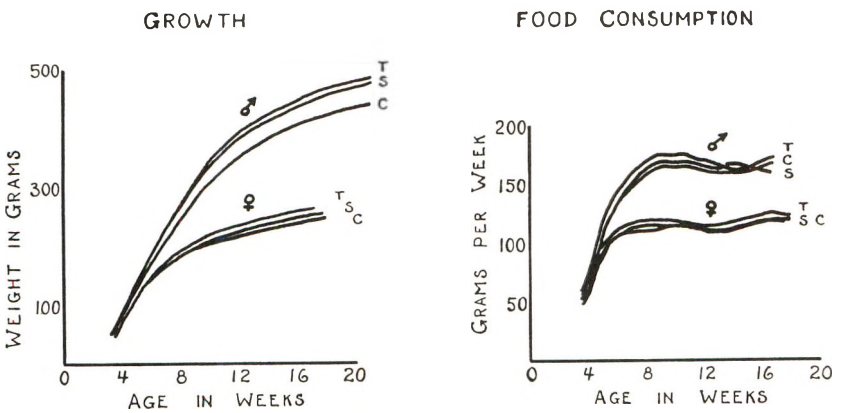

Fig. 1 Growth and food consumption of control (C), terramycin- (T) and streptomycin- (S) supplemented rats.

failures encountered during the second period may have been due to: (1) decreased reproductive performance concomitant with increased age, (2) disease, or (3) a combination of both. There was an indication, however, of a possible protective action from terramycin. During the second reproduction period of the first generation, 9 females on the streptomycin diet and 4 females on the control diet were sterile as defined earlier; however, there were only two failures of this type among the females on the terramycin diet. There was no significant difference between average birth weights of the young on any diet, and these weights were well above the minimum range for survival (5.0 to 5.4 gm) as defined by

TABLE 1

Summary of reproduction data

DIET	NUMBER OF		AVERAGE NUMBER OF YOUNG BORN			AVERAGE BIRTH WEIGHT OF YOUNG	
	Dams	Litters [1,2]	Dead	Alive	Alive 3rd day	Male	Female
						gm	*gm*
			First generation				
First reproduction							
Terramycin	30	29	0.4	11.7 ± 0.6 [3]	11.3 ± 0.6 [3]	6.5 ± 0.1 [3]	6.1 ± 0.1 [3]
Streptomycin	30	28	1.1	10.3 ± 0.7	9.9 ± 0.7	6.7 ± 0.1	6.2 ± 0.1
Control	30	28	0.9	12.0 ± 0.3	11.5 ± 0.4	6.4 ± 0.1	5.9 ± 0.1
Second reproduction							
Terramycin	30	23 [4]	1.1	11.1 ± 0.9	10.3 ± 0.9	6.7 ± 0.2	6.2 ± 0.1
Streptomycin	30	14	1.3	11.4 ± 0.9	11.1 ± 0.9	6.8 ± 0.2	6.2 ± 0.2
Control	30	17	0.7	9.6 ± 1.2	9.6 ± 1.1	6.6 ± 0.3	6.1 ± 0.3
			Second generation				
First reproduction							
Terramycin	16	16	0.3	11.1 ± 0.8	10.8 ± 0.8	7.0 ± 0.2	6.7 ± 0.1
Streptomycin	18	18	0.4	10.9 ± 0.9	10.8 ± 0.9	6.7 ± 0.2	6.4 ± 0.1
Control	14	12	0.3	11.3 ± 1.4	10.4 ± 1.4	6.4 ± 0.1	5.9 ± 0.1

[1] One or more young born alive.

[2] Unsuccessful matings involve: inability of female to conceive with three successive fertile males; death of female during pregnancy or parturition; or all young stillborn.

[3] Standard error of the mean.

[4] Difference in number of litters between terramycin- and streptomycin-supplemented rats significant at $p < 0.02$ level.

Sica and Cerecedo ('48). Only two second generation females on the control diet failed to become pregnant.

Lactation (see table 2). The primary lactation index used was the weight gain of the young from the third to the 14th day, during which time the dam's milk was their sole source of nourishment. During the first lactation period, male and female young of dams on the terramycin diet gained significantly more weight than did their counterparts on the control diet. The mean gain for the males was 2.7 gm more and for the females 3.3 gm more. The difference in gains was significant at the 0.01% level. No significant difference existed between the streptomycin and control groups.

The data show an average daily ad libitum food consumption per dam of 40.8 gm during the third to 14th days post partum. The intake for the same rats before parturition was 17.9 gm. The figure, 40.8 gm, is the average value for all diets over a lactation period during which time the food consumption was constantly increasing. There was no significant difference in the food consumption of dams on the different diets during the lactation period.

A continued effect of lactation on the growth of the young is the combined intake of milk from the dam and the early eating of food during the 14th to the 21st day of life. During this time, the male and female young on the antibiotic-supplemented diets showed significantly greater gains than their counterparts on the control diet. The males and females on the terramycin diet and the females on the streptomycin diet were significantly heavier than their counterparts on the control diet by 8.2, 8.4, and 3.3 gm, respectively, at weaning.

Because of the high percentage of breeding failures during the second reproduction period, it is difficult to make a statistical analysis. In general, however, it may be said that the antibiotics continued to exert a beneficial lactation effect on the young. The values obtained with a much smaller number of animals on the control and streptomycin diets during the second period have been noticeably raised over

TABLE 2

Summary of lactation data

DIET	NUMBER OF YOUNG AT WEANING	AVERAGE GAIN IN WEIGHT FROM 3RD TO 14TH DAY OF AGE		FEED EATEN BY DAM 3RD TO 14TH DAY	AVERAGE GAIN IN WEIGHT 14TH TO 21ST DAY		AVERAGE WEIGHT AT WEANING 21 DAYS OF AGE	
		Male	Female		Male	Female	Male	Female
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
First generation								
First lactation								
Terramycin	150	23.4 ± 0.6 [1,2]	23.0 ± 0.5 [1,2]	...	22.7 ± 0.5 [1,2]	21.1 ± 0.4 [1,2]	55.5 ± 1.1 [1,2]	52.9 ± 0.9 [1,2]
Streptomycin	141	21.2 ± 0.6	20.6 ± 0.6	...	20.3 ± 0.6 [2]	18.6 ± 0.4 [2]	50.2 ± 1.1	47.8 ± 0.9 [2]
Control	162	20.6 ± 0.4	19.7 ± 0.3	...	17.8 ± 0.6	16.4 ± 0.5	47.3 ± 0.9	44.5 ± 0.8
Second lactation								
Terramycin	108	24.3 ± 0.6	23.3 ± 0.5	455 ± 10 [1]	23.5 ± 0.7	21.6 ± 0.6	57.3 ± 1.2 [2]	54.1 ± 1.2
Streptomycin	72	24.7 ± 0.7 [2]	24.6 ± 0.5 [2]	456 ± 14	23.3 ± 0.9	22.6 ± 0.7 [2]	59.4 ± 1.4 [2]	56.5 ± 1.1 [2]
Control	69	22.3 ± 0.5	21.8 ± 0.5	435 ± 9	21.5 ± 0.6	19.8 ± 0.7	54.0 ± 1.1	51.3 ± 1.3
Second generation								
First lactation								
Terramycin	..	23.1 ± 0.7 [2]	22.2 ± 0.9 [2]	...	24.8 ± 0.8 [2]	22.9 ± 0.8 [2]	58.0 ± 1.5 [2]	54.6 ± 1.1 [2]
Streptomycin	..	20.7 ± 0.6 [2]	19.9 ± 0.6 [2]	...	22.2 ± 0.7 [2]	21.1 ± 0.5 [2]	52.3 ± 1.2 [2]	50.0 ± 0.9 [2]
Control	..	18.9 ± 0.6	17.9 ± 0.7	...	15.8 ± 0.8	15.5 ± 0.9	43.1 ± 1.2	41.8 ± 1.1

[1] Standard error of the mean.

[2] $p < 0.01$ when compared to the control group of the same lactation period.

the values obtained during the first. This may be partly due to a naturally-occurring, selective elimination of the weaker dams and young.

Second generation effects. In general, the second generation data supported the findings of the first generation in that there was no significant difference in reproductive performance, and that there was a positive effect of antibiotics on lactation, i.e. the weight gain of young from the third to 14th days and 14th to 21st days post partum, with consequent

TABLE 3

Excretion of antibiotics in feces

DIET	PERIOD [1]	PURE ANTIBIOTIC BASE		
		Intake	Feces	Excreted
		mg	*mg*	%
Terra-	A	84.4	70.8	83.9
mycin	B	87.6	73.2	83.6
	C	99.0	73.7	74.4
	Total	271.0	217.7	Av. 80.3
Strepto-	A	71.8	28.7	40.0
mycin	B	79.0	28.4	35.9
	C	76.0	27.8	36.6
	Total	226.8	84.9	Av. 37.4

[1] Four rats for 4 days in each period.

significant increases in weaning weights of terramycin- and streptomycin-supplemented young as compared to their control diet counterparts.

Excretion of active antibiotic in the feces. The quantity of active antibiotic excreted in the feces was measured for 48 rat-days on each diet (see table 3). An average of 80.3% of the terramycin but only 37.4% of the streptomycin fed was so excreted. No attempt was made to determine the fate of either antibiotic not appearing actively in the feces.

Fat content of the livers. There was no significant difference in the liver fat content of male or female rats on the three diets (see table 4). Fatty infiltration of the liver has

been attributed to several antibiotics, but mainly aureomycin and terramycin. A summary of these reports, primarily observations on hospitalized human subjects, appeared in Nutrition Reviews ('52). The contrast between our findings and other reports may be ascribed to: (1) species differences, or (2) that the observations in the human patients were artifacts of their physical conditions.

TABLE 4

Fat content (ether extract) of dry liver

DIET	TERRAMYCIN	STREPTOMYCIN	CONTROL
	%	%	%
Males [1]	7.77 ± 0.40 [2]	7.44 ± 0.41	8.04 ± 0.35
Females [1]	7.21 ± 0.41	7.35 ± 0.55	8.08 ± 0.56

[1] Average of 10 males or 15 females on each diet.

[2] Standard error of mean.

SUMMARY

Groups of 10 male and 30 female weanling rats were each fed one of three diets: a natural basal, basal plus 0.04% terramycin or basal plus 0.04% streptomycin.

The animals were bred at 4 months of age and again one month after weaning their young. There were no significant differences in reproductive ability between rats fed the three diets. Weight gains of the offspring of animals fed terramycin and streptomycin supplements during the third to 14th days post partum (lactation performance) were in general significantly increased over weight gains of young from dams fed the control diet. From the 14th to 21st days (effect of mother's milk plus food), there was in general a continued significant increase in weight gain of antibiotic-supplemented male and female young. A smaller second generation confirmed the results of the first.

There was no significant difference in average food consumption of dams on different diets during the lactation period. Thirty-seven per cent of the consumed streptomycin and 80% of the terramycin were eliminated in active form

in the feces. Neither antibiotic had any significant effect on the fat content of the livers of rats of either sex.

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NET PROTEIN VALUE OF BLOOD FIBRIN FOR THE ALBINO RAT: EVALUATION OF NITROGEN BALANCE AND CARCASS ANALYSIS METHODS

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The classical method for the determination of net protein value (n.p.v.) is multiplication of biological value, determined by nitrogen balance, by the coefficient of true digestibility of the protein (Mitchell, '48). This is an accurate and satisfactory method for assessing the relative amount of dietary protein actually utilized under the experimental conditions employed.

Recently Bender and Miller ('53) have proposed an ingenious short cut, employing carcass analysis rather than nitrogen balance, for the determination of net protein value. The theoretical basis of their proposal is identical to that of the Thomas-Mitchell nitrogen balance procedure, but their brief presentation leaves several questions unanswered with respect to the practical procedure. It was the object of the current investigation to determine the effect of previous treatment of the animals on the net protein value and to compare this value, and its reliability, with that obtained by the more laborious but well-tested nitrogen balance technique.

METHODS

Except for the commercial stock ration, all diets employed were equalized for fiber (2%) and ether extract (10%). Complete vitamin and mineral mixes were included and the

major energy source was a mixture of sucrose (10%), and glucose and dextrins in equal proportions to make 100% after allowance for the designated amount of protein for each diet.

The biological value and true digestibility of a commercial blood fibrin (Armour) were determined by the Thomas-Mitchell procedure as currently employed in this laboratory (Forbes and Yohe, '54). Ten weanling male albino rats of the Sprague-Dawley strain were fed for two weeks on the test diet (10% protein from blood fibrin), followed by two weeks of feeding on the 4% whole egg protein standardizing diet. Nitrogen balances were run on individual rats during the last 7 days of each feeding period, and the biological value, true digestibility of protein and net protein value were calculated in the usual fashion.

The method of Bender and Miller, with some modifications, was also employed for the determination of net protein value. Thirty weanling male albino rats were employed in this part of the study. Ten were placed on each of the following diets for a 7-day preliminary period: stock ration (28% protein), 10% whole egg protein, and 4% whole egg protein. Food intake was controlled at 6.0 gm per rat daily, as it was in the Thomas-Mitchell procedure. At the end of this preliminary period 5 rats from each diet were placed on the test protein diet containing 10% blood fibrin protein while the remaining 5 animals were placed on a nitrogen-free diet. Assignment of rats to the test and control diets was made by careful pairing on the basis of body weight. After 10 days on this regimen, the animals were etherized, the gastrointestinal tract was cleaned, and carcasses were ground for analysis for total nitrogen and water. Chemical analyses were made by standard procedures (Forbes and Yohe, '54). The net protein value was calculated according to Bender's equation, n.p.v. $= \frac{B_f - B_k + I_k}{I_f}$ where B_f and I_f equal carcass nitrogen and nitrogen intake of animals on the test diet, and B_k and I_k equal carcass nitrogen and nitrogen intake on the control diet.

In a second paper Bender and Miller ('53a) proposed to eliminate the carcass nitrogen analysis by reliance upon a predetermined ratio of nitrogen to water in the carcasses. Thus, only the water content of the carcass need be determined, and by use of the ratio, carcass nitrogen could be calculated. The data obtained in this study afforded opportunity to investigate the reliability of this technique.

TABLE 1

Net protein values of blood fibrin as determined by nitrogen balance and by carcass analysis methods

PRETREATMENT	NO. OF ANIMALS	NET PROTEIN VALUES AND THEIR STANDARD ERRORS		
		Thomas-Mitchell	Carcass analysis methods	
			Nitrogen analysis	Water analysis
		%	%	%
7 days on each diet prior to determination of nitrogen balance [2]	10	76.6 ± 1.57 [1]		
Stock ration	5		77.0 ± 3.97 [2]	79.1 ± 7.12 [2]
10% egg	5		78.0 ± 3.12	77.6 ± 2.71
4% egg	5		76.5 ± 3.08	85.7 ± 4.48
Total	15	Average	77.2 ± 1.83	80.8 ± 2.88

[1] Standard error.

[2] The two diets were those designated in the text as 10% blood fibrin and 4% whole egg protein.

RESULTS

The net protein value determined by the usual nitrogen balance technique averaged 76.6% ± 1.57.[1] Table 1 shows the results obtained by the carcass analysis principle of Bender and Miller. Since there is obviously no difference in the mean value or standard error between the data from carcass nitrogen analysis of the rats on the various pretreatments, all 15 values were averaged, giving a general mean of 77.2% ± 1.83. The data obtained by means of the nitrogen/water ratio of the carcasses are more variable, the general mean being 80.8 ± 2.28.

[1] Standard error.

DISCUSSION

The different pretreatments were employed with the carcass analysis method to ascertain the effect of previous protein nutriture on the results obtained. The data show that neither excess protein of good quality nor a moderate deficiency of excellent quality protein affects the results obtained by the carcass nitrogen method. The data obtained by the carcass water method are more variable and tend to give high results if the animals are moderately deficient in protein at the start of the test. The general mean of the net protein values obtained from the carcass water method is significantly ($p = 0.04$ by Student's "t" test) greater than by the carcass nitrogen method.

The successful application of the carcass analysis method depends greatly on adequate pairing of the animals designated for test and control rations, since the assumption must be made that the difference in nitrogen content of the two animals at the end of the test is the result solely of dietary treatment and not of initial difference in nitrogen content. Handled thus on an individual basis one may obtain data that are more susceptible of statistical interpretation than those obtained by the group feeding method described by Bender and Miller.

The excellent correspondence in results obtained by the nitrogen balance and carcass nitrogen methods indicates that the latter, with its saving of time and labor, has much in its favor. It should be noted, however, that 30 animals were required (this might be reduced to 20 by using one control group) to obtain by the carcass analysis method a standard error of the same magnitude as that obtained with 10 animals in the nitrogen balance procedure. It appears that the greatest accuracy is obtained by the nitrogen balance procedure in which each animal serves as his own control and in which analysis of feed, feces, and urine for nitrogen is inherently more accurate, because of greater ease of sampling, than is the analysis of rat carcasses for nitrogen.

We found the 100 N/H_2O ratio to be 4.81 $\pm$ 0.114 (s.d.) in our rats. This is a higher ratio than the 4.05 obtainable by use of Bender's regression equation expressing the relationship between N/H_2O ratio and age of the animal. The difference may be a reflection of strain difference, since we employed albino rats while Bender and Miller used a hooded variety. Of greater significance than this is the standard error of the estimate of carcass nitrogen calculated from carcass water content. The average percentage of nitrogen in the rat carcasses was 3.25 $\pm$ 0.087 (s.d.) by direct analysis, and 3.25 $\pm$ 0.128 by calculation from water content. The standard error of the estimate amounted to 0.08. An error of 0.08% in the nitrogen content of a rat carcass will cause an error of 20% in the net protein value under the conditions of this experiment. This variation could doubtless be reduced by carefully standardizing the animals by pretreatment with a diet containing an amount of good quality protein that is barely adequate, thus tending to provide animals of uniform nutritional condition at the start of the test period. In spite of our custom of considering the composition of the fat-free tissue of an animal body to be a constant it does not appear reasonable to expect reliance upon such constancy to yield results as accurate as the direct determination of the tissue constituent of interest.

SUMMARY

The net protein value of a commercial blood fibrin fed at a 10% level to young male albino rats was found to be 76.6% $\pm$ 1.57 by the Thomas-Mitchell nitrogen balance technique. A shorter method employing carcass analysis yielded a result of 77.2% $\pm$ 1.83 when carcass nitrogen was determined directly and 80.8% $\pm$ 2.88 when carcass nitrogen was calculated from carcass water analysis and a predetermined N/H_2O ratio.

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EFFECT OF ENERGY INTAKE ON THE BIOLOGICAL VALUE OF PROTEIN FED TO RATS

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In an excellent discussion entitled, "Biological Methods of Measuring the Protein Value of Feeds," Mitchell ('43) has stated, ". . . since the amino acid requirements for maintaining the nitrogenous integrity of the tissues are probably simpler than those for the construction of new protein molecules . . . it is to be expected that in general the utilization in metabolism of dietary nitrogen will be greater for maintenance than for the productive functions." Later this concept was modified (Mitchell and Beadles, '50) in view of evidence that lysine requirements are more intense for the young growing rat, while methionine-cystine requirements are more intense for maintenance of nitrogen equilibrium of the adult animal. It was thus recognized that the identity of the limiting amino acids in a protein would govern the effect on biological value of a shift in proportionate needs for maintenance and growth. No reports have been found by the authors, however, in which this concept has been tested using animals of similar age as experimental subjects.

The object of this experiment was to investigate the effect on biological value for the rat of varying the energy intake of proteins deficient in methionine or in lysine. Soybean oil meal was selected as representative of the former deficiency and blood fibrin supplemented with methionine was expected to be moderately deficient in lysine.

METHODS

Thirty weanling male albino rats of the Sprague-Dawley strain were randomized into 6 groups of 5 animals each and placed on experiment in accord with the schedule shown in table 1. The diets were semi-purified, complete in all known nutrients required by the rat except for variations in protein source, and all were designed to contain 4.0% minerals, 2.0% fiber and 10.0% ether extract. The two test diets contained, as shown by analysis, 1.60% nitrogen, while the standardizing diet contained 0.67% nitrogen. Biological values were determined by the nitrogen balance procedure customarily utilized in this laboratory (Forbes and Yohe, '54). Each period was

TABLE 1

Plan of experiment

RATS DAILY FOOD (GM)	1 to 5 4.0	6 to 10 6.0	11 to 15 8.0	16 to 20 4.0	21 to 25 6.0	26 to 30 8.0
Period 1	Soybean oil meal diet			Blood fibrin plus methionine diet		
Period 2	Whole egg protein diet			Whole egg protein diet		
Period 3	Blood fibrin plus methionine			Soybean oil meal diet		

of 14 days duration, the last 7 of which constituted the collection period. The data were treated statistically according to the analysis of variance; the authors are pleased to acknowledge the invaluable aid of M. H. Bert in this portion of the work.

RESULTS

The level of food intake had no significant effect on the endogenous nitrogen excretion per gram of body wt.[3/4] or on the metabolic N of the feces per gram of food consumed. The average figures for these types of nitrogen excretion are 0.659 ± 0.022 (s.e.) and 1.34 ± 0.022, respectively.

Table 2 shows the biological values obtained in this study, together with their standard errors and the average daily weight gain of the animals during the collection periods.

From an analysis of variance of the data for biological value it may be concluded that protein source had a "highly

significant'' effect on biological value, i.e., blood fibrin plus methionine was superior to soybean oil meal, and that this superiority existed at all food levels in both periods. The level of food intake also had a ''highly significant'' effect on biological value, an effect which was present with both proteins and in both periods. There is no evidence of significant interaction between protein source, period, or food level.

It appears obvious from a study of table 2 that the biological values obtained at 4.0 gm intake are lower than those obtained at 6.0 or 8.0 gm intake. To ascertain whether or not

TABLE 2

Biological value and average daily weight gain of rats receiving different levels of energy intake

RATS	FOOD INTAKE	PERIOD 1		PERIOD 3	
		Biological value	Wt. gain	Biological value	Wt. gain
	gm	*%*	*gm*	*%*	*gm*
		Soybean oil meal		*Fibrin plus methionine*	
1 to 5	4.0	56.7 ± 2.57 [1]	0.1	68.7 ± 3.70	0.2
6 to 10	6.0	68.6 ± 2.78	0.8	94.9 ± 2.88	1.3
11 to 15	8.0	70.0 ± 2.35	1.3	98.5 ± 1.00	2.2
		Fibrin plus methionine		*Soybean oil meal*	
16 to 20	4.0	79.8 ± 8.05	0.2	61.4 ± 3.90	0.0
21 to 25	6.0	97.7 ± 1.00	1.6	70.5 ± 2.95	0.7
26 to 30	8.0	96.2 ± 1.33	2.5	74.9 ± 2.15	1.5

[1] Standard error.

a difference existed between biological values obtained at the two higher intake levels, the data from these levels alone were subjected to an analysis of variance. These data again demonstrate the superiority of blood fibrin plus methionine over soybean oil meal and also show that the biological values obtained for these proteins did not vary significantly when the food intakes of 6.0 and 8.0 gm daily were compared.

DISCUSSION

From the growth data in table 2 it may be seen that the animals receiving 4.0 gm of food daily grew very slowly. It

is significant that even at these very low rates of gain the superiority of one protein over the other is evident. The ratio of gains of rats receiving methionine-supplemented fibrin to those receiving soybean oil meal approximates 2:1 at all levels of energy intake. Thus, while the relative gains between protein sources are similar at all levels of food intake, the absolute difference is greater on the higher intake. The coefficient of variation of the gains likewise decreased as the rate of gain increased. These observations point out some advantages of attaining good growth rates in feeding experiments and show that an experiment need not be considered invalid because of poor growth rate.

It is probable that the animals on the lowest energy intake utilized much of their absorbed protein to supply energy for maintenance, and hence could not utilize it for tissue synthesis. This would adequately account for the lower biological values obtained with the animals on the lowest energy intake since the nitrogen of the protein utilized for energy would appear in the urine as waste nitrogen. This demonstrates the necessity of adequate energy intake in the determination of the biological value of protein.

All animals were in positive nitrogen balance throughout the three collection periods. This shows that sufficient energy and protein were consumed to meet the maintenance requirements for these nutrients and that body reserves of protein were not called upon for their energy value.

The similarity in biological values obtained at intakes of 6.0 and 8.0 gm of food indicates that at both levels the protein intake was not above the optimum in relation to the energy intake. Thus, the animals were able to utilize each protein at the maximum rate of efficiency characteristic of the mixture of amino acids absorbed. In other words, the energy intake was sufficient to support greater growth than was obtained, but the net available protein limited the growth. Hence, conditions were optimum for most efficient utilization of the protein supplied.

The relative methionine deficiency of soybean oil meal might be expected to cause a lower biological value for the slower-growing animal if it is correct that the methionine requirement for maintenance is more intense than for growth. That this was not observed between the food intake levels of 6.0 and 8.0 gm is probably a result of the relatively small difference in the percentage of total nitrogen which was used for maintenance at the 6.0 and 8.0 gm levels of food intake as shown in table 3.

TABLE 3

Nitrogen appearing in gain and used for maintenance

RATS	FOOD INTAKE	PERIOD 1		PERIOD 3	
		Nitrogen balance	Maintenance nitrogen	Nitrogen balance	Maintenance nitrogen
	gm		*mg/day*		*mg/day*
		Soybean oil meal			
1 to 5	4.0	13.14	19.18	22.14	20.54
6 to 10	6.0	35.89	23.76	63.17	28.22
11 to 15	8.0	51.42	29.42	88.13	36.68
		Blood fibrin plus methionine		*Soybean oil meal*	
16 to 20	4.0	28.52	22.35	12.44	23.43
21 to 25	6.0	68.67	26.39	32.64	29.39
26 to 30	8.0	93.03	30.63	52.81	35.61

Blood fibrin supplemented with methionine was chosen for investigation with the expectation that it would then be more deficient in lysine for a rapidly growing rat than for one growing slowly. The fact that methionine supplementation overcame essentially all of the amino acid deficiencies of blood fibrin, yielding biological values of 95% or better, indicates that the lysine content of the protein was adequate under the conditions of this experiment.

Table 4 presents a summary of estimated essential amino acid requirements expressed as percentages of the total essential amino acids required by young and by adult rats together with similar data on the composition of the proteins used in this study. All values were computed from data obtained in the sources indicated. The high values estimated by

TABLE 4

Proportionate requirements for essential amino acids as determined for young and for adult albino rats and proportionate concentrations of these amino acids in the proteins investigated in this study

AMINO ACID [1]	YOUNG RATS (Growth) (Amino acids diets) (Rose at al., '49)	ADULT RATS (N-balance) (Amino acids diets) (Benditt et al., '50)	ADULT RATS (N-balance) (Protein diets) (Mitchell, '50)	BLOOD FIBRIN (Block and Mitchell, '46)	SOYBEAN OIL MEAL (Block and Mitchell, '46)
Lysine	17.2	7.8	5.6	13.6	13.3
Histidine	6.8	3.8	3.9	4.2	5.3
Arginine	3.4	..	11.4	12.0	16.3
Phenylalanine plus tyrosine	15.6	6.5	20.5	9.3	13.1
Tryptophan	3.4	3.8	2.9	5.3	2.8
Methionine plus cystine	10.4	11.0	9.8	4.8	4.6
Threonine	8.6	11.0	6.3	12.2	9.2
Leucine	13.8	13.8	17.1	21.7	15.1
Isoleucine	8.6	27.5	10.6	7.7	10.8
Valine	12.0	14.8	12.0	9.3	9.6

[1] Data in this table indicate percentage of total essential amino acids required as the specific amino acids.

Mitchell may be explained by recognizing that these represent the minimum amounts of these particular amino acids (arginine, phenylalanine plus tyrosine, and leucine) in the proteins employed in his experiments. The excellent quality of fibrin supplemented with methionine indicates that the requirements for the growing rat estimated by Rose et al. ('49) may be excessive in the case of lysine and of phenylalanine plus tyrosine. Comparing the data of Rose and of Benditt et al. ('50), young growing rats (or fast growing) would impart lower biological value to a protein than would adult (or slow growing) rats if the protein were low in lysine, histidine, arginine or phenylalanine. The reverse would be true if protein were deficient in isoleucine. Young and adult rats would give similar values in the case of deficiencies of other essential amino acids.

SUMMARY

Biological values of soybean oil meal protein and of methionine-supplemented blood fibrin were determined with 30 weanling male albino rats by the nitrogen balance procedure. Each protein was incorporated into otherwise complete diets at a 10% level. Food intake was regulated at 4.0, 6.0 or 8.0 gm daily. It was found that at 4.0 gm food intake the biological value of soybean protein and of methionine-supplemented fibrin was 59.1 and 74.3, respectively. At 6.0 and 8.0 gm of food intake the values did not vary significantly between intake levels and averaged 71.0 and 96.8 for the two proteins. The low biological values obtained at 4.0 gm intake are ascribed to utilization of a portion of the dietary protein for energy to support weight maintenance of the animals.

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INJURY TO GUINEA PIGS THAT FOLLOWS A HIGH INTAKE OF PHOSPHATES

THE MODIFYING EFFECT OF MAGNESIUM AND POTASSIUM [1]

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Hogan and Ritchie ('34) had some degree of success in rearing guinea pigs on a semi-synthetic diet when the ration contained 15% of cellophane but the animals failed almost completely when the amount was reduced to 3%. The importance of bulk was also emphasized by Booth, Elvehjem and Hart ('49) who reported that gum arabic was decidedly superior to cellulose. Some workers do not regard the difference as important and Reid and Briggs ('53) replaced gum arabic with cellophane, with no evident loss in growth-promoting activity. Wooley ('54) used cellophane as a source of bulk in the rations of rabbits and presumably he thought it was not inferior to gum arabic for that purpose.

According to Roine, Booth, Elvehjem and Hart ('49) diets for guinea pigs were improved by including in them considerably more magnesium and potassium than had been customary. Wooley ('54) found that the rate of growth in rabbits was notably accelerated by increasing the amount of potassium in the diet. In a subsequent report, however, Wooley and Mickelsen ('54) stated that some of their basal diets were improved to about the same degree with either

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[2] Taken from a thesis to be presented by W. B. House to the Graduate School of the University of Missouri.

sodium or calcium and either seemed somewhat superior to potassium. They also stated that the requirement of the rabbit for sodium, potassium and calcium was complicated by the percentages of protein and fat in the diet.

Hogan, Regan and House ('50) observed that certain maladjustments in the intake of calcium and phosphorus by guinea pigs were followed by wrist soreness, joint stiffness and deposits of calcium phosphate. Small, white pinhead swellings on the pads of the feet or on the top or side of the toes are often the first indication of the deposits. In more severe cases deposits are found on the elbows, ribs, spinal column, in the subcutaneous connective tissue and on the stomach and duodenum. Hogan et al. reviewed the literature on this topic and pointed out that this syndrome is similar to the one first described by Wulzen and Bahrs ('41). Our more recent observations were described by Hogan, House and Regan ('54).

The investigations of other workers are limited almost entirely to the requirement for growth. This report is concerned with the effect of gum arabic and of larger intakes of magnesium and potassium on the development of wrist stiffness and on deposits of calcium phosphate. The procedure was described by Hogan and Hamilton ('42). The average initial weight of the experimental animals was approximately 200 gm.

EXPERIMENTAL

Series I. The results described by Hogan, House and Regan ('54) led us to believe that large amounts of dietary phosphorus are harmful and that under some circumstances the ratio of calcium to phosphorus is important. In series I therefore, calcium and phosphorus were included in the diets at different levels and with different ratios. One ration contained twice as much calcium as phosphorus. Some of them contained twice as much phosphorus as calcium. Some contained intermediate amounts. The mineral mixtures used in this series are not described in detail, in order to save space,

but the amounts of calcium and phosphorus were adjusted to give the desired percentages and ratios. All other mineral elements were present in these salt mixtures in about the same proportion as in salt mixture V (Richardson and Hogan, '46). Gum arabic, magnesium and potassium were included in these rations in the proportions recommended by Roine et al. ('49). The constituents of the rations were analyzed for calcium, magnesium, potassium and phosphorus, and the percentages of these minerals in the various diets were calculated from the analyses. The diets are described in tables 1 and 2 and the results are summarized in table 2.

It will be noted that there are large differences between the rations in the amounts of calcium and phosphorus they contain, and in the ratios in which these elements are present. However, there were no extreme differences between the results that were obtained. There were no mortalities in any group and the animals on all rations gained in weight at approximately the same rate. There were definite variations in the time required for symptoms to appear, although the differences were smaller than we had expected. Ration 2651 contains comparatively small amounts of calcium and phosphorus and the appearance of stiffness was delayed until about 23 weeks. Rations 2653, 2666 and 2854 contain less than one part of calcium to one of phosphorus and the animals that consumed them developed stiffness quickly. Rations 2652 and 2667 contain large amounts of both calcium and phosphorus, and they have a high calcium to phosphorus ratio. Animals on these diets developed stiffness more slowly than did those on the three rations just mentioned which contained an excess of phosphorus. The amount of inorganic phosphorus in the blood of animals on rations 2653, 2666 and 2854 was somewhat above the normal range. The amount was normal on the other three rations. The amount of calcium in the serum was normal on all rations. Only one animal, on ration 2652, developed mineral deposits.

The animals described in table 2 were injured less severely than was expected by consuming an excess of phosphorus.

TABLE 1

Constituents that were present in the same amounts in all diets

Casein [1]	30.00 gm	Vitamin A [2]	1475 I.U.	Ca-pantothenate	3 mg
		Vitamin D	320 I.U.	Niacin	10 mg
Soybean oil	4.00 gm	Vitamin E	12 mg	Choline Cl	300 mg
Magnesium oxide	0.55 gm	Menadione	0.2 mg	Inositol	200 mg
Potassium acetate	2.74 gm	Thiamine HCl	1.0 mg	p-Aminobenzoic acid	10 mg
		Riboflavin	1.4 mg	Biotin	40 μg
		Pyridoxine HCl	1.0 mg	Folic acid	300 μg

Ascorbic acid, 10 mg daily per animal, supplied separately.

Magnesium 0.4% approximate.

Potassium 1.5% approximate.

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

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

All vitamins except ascorbic acid were intimately mixed with the other constituents.

Guinea pigs described in an earlier publication (Hogan, House and Regan, '54) were given a ration that contained 0.9% of calcium and 1.7% of phosphorus and they were markedly affected. Gains in weight were low, the mortality rate was high, and 50% of the animals developed mineral deposits. The results in table 2 are almost completely at variance with our previous experience. In our earlier work

TABLE 2

The effects of diets that contain varying amounts of calcium and phosphorus
All diets contain gum arabic and added magnesium and potassium

DESCRIPTION OF DATA		RATION					
		2651	2854	2652	2653	2666	2667
Variables in the diets							
Sucrose	gm	43.9	42.6	42.6	41.5	39.6	39.0
Salt mixture	gm	3.8	5.1	5.1	6.2	8.1	8.7
Calcium	%	0.67	0.60	1.14	1.14	1.14	2.28
Phosphorus	%	0.70	1.19	0.72	1.31	1.74	1.25
Observations on the animals							
Number and sex		5♂5♀	3♂2♀	3♂3♀	3♂3♀	2♂3♀	3♂2♀
Av. daily gain, first 12 weeks	gm	3.8 3.0	3.8 1.9	2.7 3.0	2.8 2.6	2.2 3.5	3.5 3.3
Av. daily food consumption for 12 weeks	gm	17.3	16.6	16.6	17.1	17.4	19.2
Stiffness and mineral deposits							
No. that became stiff, (32 weeks)		4	2	5	3	3	4
Av. time for stiffness to develop, weeks		23	5	10	5	8	12
No. with calcium phosphate deposits		0	0	1	0	0	0
Av. time for deposits to develop, weeks		..	0	23	..	..	..
Av. inorganic P in the blood [1] over a 32 week period	mg %	5.7	7.2	5.6	6.6	7.5	5.4
Av. calcium in the serum	mg % [2]	12.4	..	12.8	13.5	13.2	13.5

[1] Averages of 40 or more determinations.
[2] Averages of three to 5 determinations.

the amounts of calcium and phosphorus and their ratios in the diet, had been of decisive importance. The data in table 2 would indicate that these factors had only a moderate effect on the response of the animals. Our search for the explanation of this discrepancy is described in the following sections.

Series II. The rations in series I contained gum arabic and added amounts of magnesium and potassium and the next series was planned to determine whether these changes were responsible for the unexpected results. The rations used in series II contained casein 30%, salts V 5%, and the vitamin mixture described in table 1. Other details and a summary of the results are shown in table 3.

It was reported in an earlier publication (Hogan et al., '54) that guinea pigs develop deposits of calcium phosphate on a ration, no. 903, which contains about 1.0% each of calcium and phosphorus. Ration 3480 of series II is similar to ration 903 in the percentages of calcium and phosphorus and the results obtained are also similar. The contrast between the results obtained on ration 3480, and on any of the rations included in table 1, is impressive. The rate of gain in weight on ration 3480 was lower than any we have yet reported, and 4 of the 9 animals checked developed calcium phosphate deposits in approximately 7 weeks. Ration 3514 contains gum arabic in place of celluflour, but otherwise it is the same as ration 3480. It seems quite certain that the substitution of gum arabic for celluflour improved the diet. The animals gained in weight more rapidly and there were no deposits of calcium phosphate. Ration 3515 contains added magnesium and potassium, but it is otherwise practically identical with ration 3480. Ration 3516 also contains added magnesium and potassium but is otherwise practically identical with ration 3514. It is immediately apparent that there was a tremendous improvement in growth rate when the amounts of magnesium and potassium were increased. All animals on rations 3515 and 3516 grew reasonably well and there were no deposits of calcium phosphate. These results indicate that the inclusion of added magnesium and potassium in the rations de-

scribed in table 2 was responsible for more rapid gains in body weight than was expected, and why the development of calcium phosphate deposits was so rare.

Series III. Ration 1237, described in an earlier publication (Hogan et al., '54), contained approximately 1.6% of phosphorus and 50% of the animals developed deposits of calcium

TABLE 3

The effects of type of bulk, and of added magnesium and potassium
All diets have a calcium : phosphorus ratio of 1 : 1

DESCRIPTION OF DATA		RATION			
		3480	3514	3515	3516
Variables in the diets					
Sucrose	gm	45.8	46.0	42.6	42.8
Celluflour [1]	gm	15.0	...	15.0	...
Gum arabic	gm	...	15.0	...	15.0
Salts V	gm	5.0	5.0	5.0	5.0
Calcium carbonate	gm	0.2	...	0.2	...
Magnesium oxide	gm	...	...	0.5	0.5
Potassium acetate	gm	...	...	2.7	2.7
Magnesium	%	0.04	0.08	0.34	0.38
Potassium	%	0.4	0.5	1.5	1.6
Calcium	%	0.9	0.9	0.9	0.9
Phosphorus	%	0.9	0.9	0.9	0.9
Observations on the animals					
Number and sex		7 ♂ 5 ♀	12 ♂ 4 ♀	12 ♂ 4 ♀	8 ♂ 8 ♀
Av. daily gain first 12 weeks	gm	1.0 1.9	2.5 2.4	3.7 3.1	5.0 3.4
Stiffness and mineral deposits					
No. of animals checked		9	7	6	15
Duration of observation period, weeks		7	17	16	6
No. that became stiff		9	7	5	4
Av. time for stiffness to develop, weeks		4	14	12	9
No. with calcium phosphate deposits		4	0	0	0
Av. time required for deposits to develop, weeks		7	..	..	..
Mortality first 12 weeks	%	33	6	25	13

[1] Purchased from the Chicago Dietetic Supply House, Inc., Chicago, Ill.

phosphate in 7 weeks. This ration imposes much more severe experimental conditions than does ration 903 on which series II was based. Series III is based on ration 1237. All diets contained casein 30%, salts V 5%, sodium dihydrogen phosphate 3.6%, and the vitamin mixture described in table 1. The variable constituents and the results are shown in table 4.

TABLE 4

The effects of type of bulk, and of added magnesium and potassium
All diets have a calcium: phosphorus ratio of approximately 0.5: 1

DESCRIPTION OF DATA		RATION			
		3475	3476	3477	3478
Variables in the diets					
Sucrose	gm	42.2	42.4	39.0	39.2
Celluflour	gm	15	...	15	...
Gum arabic	gm	...	15	...	15
Salts V	gm	5.0	5.0	5.0	5.0
Sodium phosphate NaH_2PO_4, H_2O	gm	3.6	3.6	3.6	3.6
Calcium carbonate	gm	0.2	...	0.2	...
Magnesium oxide	gm	...	...	0.5	0.5
Potassium acetate	gm	...	...	2.7	2.7
Magnesium	%	0.04	0.08	0.34	0.38
Potassium	%	0.4	0.5	1.5	1.6
Calcium	%	0.9	0.9	0.9	0.8
Phosphorus	%	1.7	1.7	1.7	1.7
Observations on the animals					
Number and sex		49 ♂	26 ♂ 4 ♀	12 ♂ 7 ♀	17 ♂ 8 ♀
Av. daily gain first 12 weeks	gm	0.4	1.5 1.5	3.6 3.1	3.2 3.2
Stiffness and mineral deposits					
Duration of observation period, weeks		15	8	10	8
No. of animals checked		16	15	16	17
No. that became stiff		..	8	1	4
Av. time for stiffness to develop, weeks		..	5	10	10
No. with calcium phosphate deposits		9	8	0	0
Av. time required for deposits to develop, weeks		9	6	..	..
Mortality first 12 weeks	%	90	53	26	16

All rations in this series contain approximately twice as much phosphorus as calcium. The bulky constituent is celluflour in ration 3475 and gum arabic in ration 3476, and in our opinion the animals were damaged slightly less on ration 3476 than on ration 3475. Three guinea pigs on a diet that contained 15% of methyl cellulose as a source of bulk were checked for calcium phosphate deposits, and visible deposits were detected in all three, in a period of approximately 8 weeks. The total mortality rate in 12 weeks was 87%. The important observation was the high incidence of calcium phosphate deposits when these rations were consumed. In all, 34 animals were checked and deposits were detected in 20 of them. Rations 3477 and 3478 contain added magnesium and potassium and again these additions brought about remarkable improvement. The mortality rate was reduced, the rate of gain was vastly improved, and no deposits of calcium phosphate were detected. The extent of the reversal in response when magnesium and potassium are included in the experimental diet can hardly be overemphasized.

It may be that gum arabic is superior to celluflour as a constituent of the diet of guinea pigs, and we were convinced at the time that there is an unrecognized nutrient in gum arabic that is important for these animals. However, analysis shows that one sample contained 0.27% of magnesium and 0.65% of potassium. It seems now that if gum arabic is superior to celluflour the difference in mineral content may be the explanation.

DISCUSSION

In earlier studies (Hogan et al., '50, '54) a large excess of phosphates in the diet had markedly reduced the rate of growth and caused a high incidence of calcium phosphate deposits. For that reason the failure to obtain a similar result in series I was unexpected. As is brought out in the second and third series, this was a consequence of including in the diet added amounts of magnesium and potassium, cations that afford a high degree of protection against stiff-

ness and mineral deposits. When the diet is low in magnesium and potassium the amount of phosphorus in the diet is a determining factor in the deposition of calcium phosphate in the soft tissues. It is possible that an excess of any acid-forming element is of major importance in the development of this abnormality.

We would like to know whether or not these observations could be applied usefully to man. As human beings advance in age, there is deterioration in the flexibility of the joints, often accompanied by soreness, and by excessive calcification around the joints, in the cardiovascular system and in the kidneys. In some individuals there is no serious deterioration, or at least not until late in life. In others the deterioration is evident at a comparatively early age. The fundamental cause of this disability, and of the variability of its occurrence, is unknown. However, in guinea pigs the appearance of the symptoms can at least be enormously accelerated by maladjustments in the mineral intake. If nutritional maladjustments are the cause of excessive calcification in one species it is reasonable to suppose they may be the cause in another. The physiological processes in man and in guinea pigs differ in some important respects, and it is possible that the maladjustment resulting in injury in one species would have no effect on the other. A better understanding of the factors that are concerned with abnormal calcification should lengthen the period of usefulness of our population.

SUMMARY

Our earlier report that the consumption of an excess of phosphates is injurious to guinea pigs, was confirmed. The symptoms are slow gains in weight, the development of stiff joints, of calcium phosphate deposits, and a high mortality rate.

The symptoms were most severe on the rations that contained calcium 0.9%, phosphorus 1.7%, magnesium 0.04%, and potassium 0.41%.

When the rations were changed to contain approximately 0.35% of magnesium and 1.5% of potassium the damage to the animals was reduced remarkably. The animals made moderate gains in weight, few of them became stiff, deposits of calcium phosphate were rare, and the mortality rate was low.

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FURTHER EXPERIMENTS ON THE UTILIZATION OF CALCIUM FROM SALTS BY COLLEGE WOMEN [1]

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ONE FIGURE

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In an earlier study made by Patton and Sutton ('52) in this laboratory on the utilization of calcium from 4 salts there were indications that the order in which the salts were taken was a factor in the utilization of calcium from these sources. In that study a subject was given one of the salts for two 7-day periods, then a second salt for the same period of time and finally a third one. It was observed that, in general, the calcium from the first salt taken was utilized to a greater percentage than subsequent ones. This posed the question as to whether or not the utilization of calcium salts by human beings was limited by the time factor. The present study was planned to give a subject the same salt for a period of 6 weeks with the objective of determining whether or not she would utilize the calcium from the same salt equally well from week to week.

Milk was given to another group for the same length of time in quantities sufficient to supply a comparable amount of calcium for comparison of utilization with that from the salts.

The data from the earlier study (Patton and Sutton, '52) also gave indication of a relationship between calcium utilization and basal metabolic rate. The present study was designed to give further consideration to this relationship.

[1] Journal Article No. 42–45, Ohio Agricultural Experiment Station.

PROCEDURE

The design of the experiment was developed by the Statistics Labortaory of The Ohio State University. Nine college women, considered by a physician to be in good health, were selected for study. The basal metabolic rates were observed and the subjects were classified into three groups on the basis of their variation from normal standards for their age, sex and body surface. In each group the basal metabolic rates of the three subjects varied from approximately 12% below the Aub-Dubois standard to approximately the predicted normal rate of the same standard. One group received milk as a supplementary source of calcium in the basal diet; the second group, calcium gluconate; and the third group, calcium carbonate.

The basal diet of the earlier study by Patton and Sutton ('52) was modified by substituting French bread for white bread in several meals during the week in order to reduce the calcium content of the daily diet to a level of approximately 300 mg. It met the recommended allowances of the National Research Council in all other nutrients except riboflavin, which was given at the 1 mg level in tablet form. The diet was further supplemented by 400 I.U. vitamin D. Seven menus were planned and repeated 8 times during the study. The first week was termed an adjustment period and no balance was determined. The customary balance techniques were followed during the remaining 7 weeks. The first experimental period was used to establish the base line for calculation of the utilization of calcium in the supplements taken during the succeeding 6 weeks. Milk supplied 305 mg of calcium and the gluconate and carbonate salts each furnished 280 mg. The basal diet plus supplements provided these subjects with a daily calcium intake of approximately 600 mg. Apples, a food low in calcium, were given ad libitum to provide additional calories for those whose appetites were not satisfied with the basal diet. All food portions were weighed to 0.1 gm and were completely consumed by all subjects.

Aliquots of 24-hour urine specimens were preserved with concentrated hydrochloric acid. Portions of the weekly composite were analyzed for calcium by the McCrudden method as modified by Stearns ('29) without preliminary ashing. Using the method of this same investigator, hydrochloric acid digests of food and feces composites were wet ashed, the calcium precipitated as the oxalate, and titrated against 0.01 N potassium permanganate.

The experiment took place in January and February so there was no appreciable loss in perspiration.

RESULTS

For a period of one week the 9 subjects were fed the basal diet which contained the daily average of 307 mg of calcium in order that they might become acquainted with the routine and make adjustments from their freely-chosen diets to the experimental diet. Collection of excreta and food began on the 8th day and continued through the 49th day. The balances for the 9 women during the 7 weeks of observation are reported in table 1. All subjects lost calcium on the basal diet, the amounts varying from 9 to 113 mg with an average of 48 mg.

By adding approximately 300 mg of calcium to the diet in the form of carbonate or gluconate salts or milk it was expected that the subjects would attain equilibrium. It will be noted from table 1 that 7 of the 9 subjects were in positive balance during the first week of supplementation. All subjects that were receiving milk and calcium carbonate in addition to the basal diet (with an average intake of 645 and 623 mg of calcium, respectively) were in positive balance. Only one of the three subjects receiving calcium gluconate was storing calcium. It will be noted that one of the subjects in the gluconate group who was losing calcium, P. R., continued to lose calcium for the duration of the study; the other, R. S., lost during 4 of the 6 periods of supplementation. The motility of the gastro-intestinal tract may have been a factor

in the case of P. R. since she passed an average of two stools per day.

The balance data for the second, third, 4th, 5th and 6th periods indicate a pattern of loss and storage which occurs with some rhythm on alternate weeks for 4 of the subjects.

TABLE 1

Calcium balances, in milligrams, of 9 college women on a basal diet and during 6 periods of supplementation with milk, calcium gluconate, and calcium carbonate

SUBJECT	PERIOD OF BASAL DIET [1]	PERIOD OF SUPPLEMENTATION [1]					
		1	2	3	4	5	6
				Milk			
J. M.	— 42	57	— 10	67	— 13	19	72
M. E. W.	— 59	41	— 66	34	— 106	— 76	2
L. Y.	— 113	30	— 80	— 37	— 90	— 21	60
				Calcium gluconate			
O. D.	— 9	47	39	— 2	30	13	72
P. R.	— 26	— 83	— 33	— 26	— 71	— 4	— 13
R. S.	— 48	— 7	— 52	49	— 64	— 44	87
				Calcium carbonate			
E. H.	— 25	62	— 29	23	— 31	25	70
R. J.	— 78	44	— 87	— 4	— 29	— 22	11
D. S.	— 34	26	— 44	20	— 71	94	0

[1] All periods were of 7 days duration.

As pointed out above, P. R. was in negative balance during the 6 periods and O. D., in the same group, was storing or in equilibrium (— 2 mg) during the 6 periods.

These data were analyzed by the Statistics Laboratory of The Ohio State University to determine the significance of the differences in balances due to form of calcium used to supplement the basal diet. The method of least squares was used to determine the significance of these differences. It was shown that the differences in balances on the three treat-

ments were not significant. However, the analysis showed that retentions varied significantly from week to week. The variations from week to week were less during the 5th and 6th weeks than during the preceding 4 weeks.

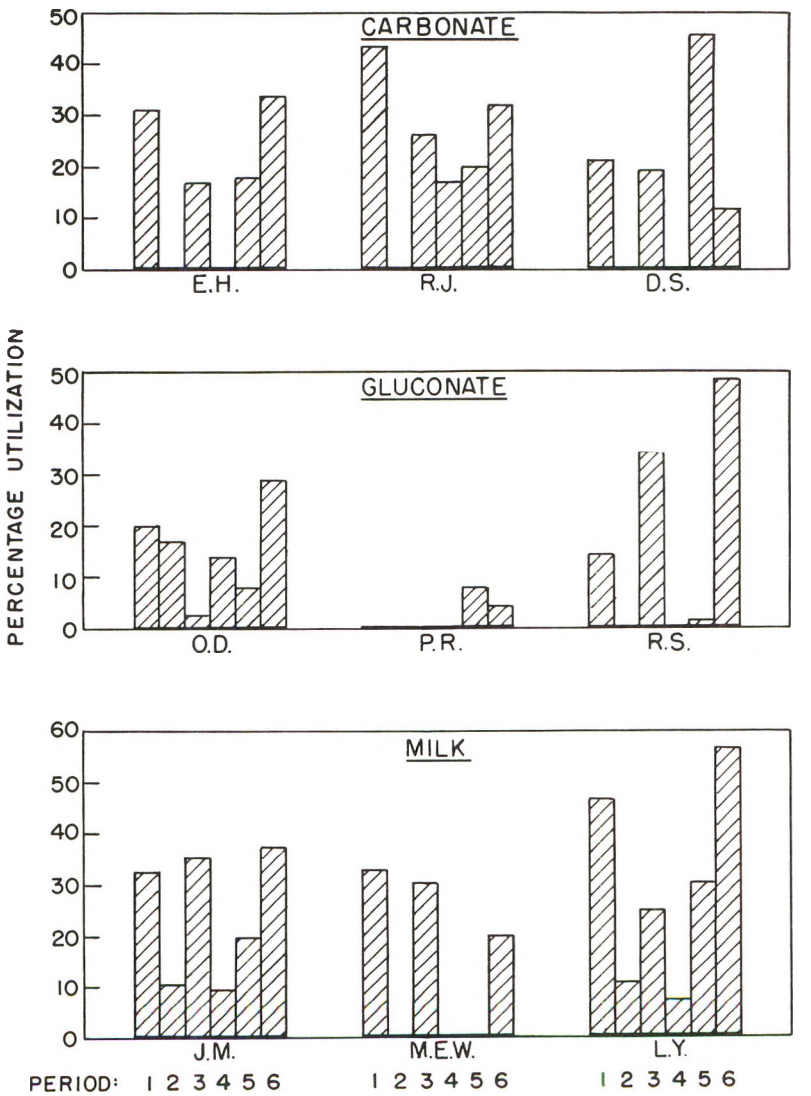

Fig. 1 Percentage utilization of calcium from milk, calcium gluconate and calcium carbonate.

The data were studied from the standpoint of utilization of the supplementary calcium, using the method of Steggerda and Mitchell ('45). The average utilization of calcium from milk for the three subjects for 6 periods was 23.7%; for carbonate, 18.8%; and for gluconate, 11.8% (fig. 1). During the earlier study by Patton and Sutton ('52) the carbonate utilization was 10.8% for 7 subjects for two periods each and gluconate utilization was 28.8% for 6 subjects for two periods each. In the present study the one subject, P. R., utilized calcium in the gluconate on the average to the extent of 2.1%; whereas O. D. utilized it to 15.0%; and R. S., to 16.5%. The average for the three is reduced because of the poor utilization by P. R.

The statistical design of the study was planned so the basal metabolic rates at the beginning of the study of the three subjects within each of the groups ranged from 12% below the Aub-Dubois standard to approximately the predicted normal rate. In other words, there was a person in each group whose rate might be said to be high, another medium, and the third low by comparison with one another. For instance, in the group receiving milk as a supplementary source of calcium, the basal metabolic rate of J. M. was 14% below the Aub-Dubois standard; M. E. W., 11% below; and L. Y., 3% below. According to commonly accepted criteria, all values would be classified as normal.

A statistical analysis of the balance data showed that the basal metabolic rate was a factor that significantly affected retention. The retention was inversely related to the basal metabolic rate — the greater the rate the less the retention. This is in contradiction to the trend indicated in the earlier study made by Patton and Sutton ('52) and warrants further investigation.

The basal metabolic rates of the subjects were made at two intervals during the time of the study, the third and 6th weeks of supplementation. The rates for all subjects except one, E. H., were lower at the close of the study than

at the beginning. The retentions of 7 of the 9 subjects were largest during the 5th and 6th periods; the remaining two subjects retained the largest amounts in the first period. Since the kind of supplement was not a factor in utilization this comparison may indicate a trend of inverse relationship as shown by the balance data using the initial basal metabolic rate. Subsequent studies of calcium utilization will be designed to obtain additional information on this relationship.

SUMMARY

The calcium balances of 9 college women consuming approximately 600 mg of calcium were studied in relation to period to period variation, source of calcium used to supplement a low-calcium diet, and the effect of basal metabolic rate on utilization.

Approximately one-half of the calcium consumed came from foods commonly served in American diets; the other half was supplied by milk to three subjects, calcium carbonate salts to three subjects and calcium gluconate salts to the remaining three. Statistical analysis of the data showed that under present experimental conditions, milk, calcium gluconate, and calcium carbonate were utilized equally well by these subjects as a source of calcium.

Differences in balance from week to week were significant but the relative importance of the time factor was reduced during the final two weeks, the 5th and 6th periods, from that in the preceding periods.

Basal metabolic rate was inversely related to calcium utilization. In general, the basal metabolic rates of the subjects were lower at the close of the study than at the beginning and this may be related to the greater utilization during the 5th and 6th periods.

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