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CYRUS EDWIN FRENCH

(1915–1960)

CYRUS EDWIN FRENCH

Cyrus Edwin French
— *A Biographical Sketch*

(June 2, 1915–January 3, 1960)

The passing of Cyrus E. French at the peak of his career was a shock to his many friends and a serious blow to the world-wide program of nutritional research in which he was actively engaged.

Born at Chesterfield, Maine, he was one of four children, the son of Joseph and Bertha Sykes French. His boyhood was spent in Boston, Massachusetts where he attended the Mechanic Arts High School prior to matriculation at the University of Massachusetts in 1934. Following his graduation from that institution in 1938, he accepted a position at the Pennsylvania State University in the Department of Animal Nutrition and in December of that year married Doris Jenkins of Shrewsbury, Massachusetts.

Though a newcomer to the research group at Penn State, his potental leadership and superior competence soon became apparent. He received the Master of Science degree and the Ph.D. degree in Animal Nutrition from the Pennsylvania State University in 1941 and 1947, respectively.

During the years at Penn State where he became Professor of Animal Nutrition he established an enviable record of accomplishment. Among his most important contributions to the science of nutrition was a series of eight papers, all published in the Journal of Nutrition, which dealt with the various aspects of fat utilization. These studies included investigations of the relationships between fat utilization and (1) calcium, (2) percentage of fat in the diet, (3) reproduction, (4) lactation, and (5) longevity.

His versatility and wide interest in nutrition is further illustrated in six papers dealing with the nutritive requirements of the white-tailed deer. This work holds a high place in its field as it represents practically the only controlled experimentation on the nutrition of this important game animal.

Dr. French was in charge of a very extensive project which involved a survey of the composition of forage crops in Pennsylvania with respect to content of ten nutrient elements including manganese, copper, boron, zinc and cobalt. This project of several years' duration involved many people and only French's versatility, diplomacy and persistence made the undertaking a success. He was intensely interested in nutrition as applied to animals, plants or to man. He participated as co-author of a textbook, Energy Metabolism and Nutrition.

Much of his work required not only vigor and competence but also patience and a cooperative spirit in working with others. His ability to get along with his associates, his sparkling good humor endeared him to all. One of his pupils once remarked to me, "I learn so much from him and he doesn't talk down to me."

In addition to the position of leadership among his associates which developed naturally, his counsel was also sought by workers in closely associated fields of study. His training in and appreciation of the priniciples of physiology, biochemistry, and nutrition resulted in a perspective that was a source of inspiration to his many associates.

His interest and activities were by no means limited to his major field of work. His great versatility and enthusiasm were evident in his avocations of woodworking, fishing, hunting, and participation in local civic affairs. With his wife and two daughters, Judith Elizabeth and Martha Belle, the family was an asset to any community. "He was a good neighbor."

During World War II he served as Executive Officer of the Nutrition Division, Army

Medical Center, Washington, D. C. and later occupied the same position at the U. S. Army Medical Nutrition Laboratory, Chicago, Illinois. In March, 1945, the defeat of the German armies in the Netherlands appeared imminent and a request was made by the Pubic Health Branch of Supreme Headquarters Allied Expeditionary Forces for a nutrition survey team to be dispatched to that area. These rapid series of one-day surveys, made at the heels of the retreating German army, yielded valuable information about local food supplies and the nutritive condition of the population. For his work in the Netherlands Dr. French was decorated by the Netherlands Government.

More extensive studies followed during succeeding months which included many clinical observations of the half-starved people of Holland and Germany. These diagnostic surveys in the war torn countries were of special value in determining what food would be most suitable in bringing the undernourished civilian population back to a satisfactory nutrient intake.

Following World War II, he assisted in summarizing and preparing the survey data which was included in the publication "Malnutrition and Starvation in Western Netherlands," edited by Dr. G. C. E. Burger, Dr. J. C. Drummond and Dr. H. R. Sandstead.

After his service overseas, he returned to the Pennsylvania State University to continue his distinguished career. At this time he had become widely known and respected by his many colleagues. On leave of absence in 1953 he spent a year at Northwestern University with the Rheumatic Fever Research Institute where he established a nutrition unit. His work there involved extensive biochemical studies of the anti-inflammatory activity of egg yolk with guinea pigs.

As a major in the U. S. Army Reserve, he kept abreast of the latest developments by participating each year in two weeks' active duty training as a mobilization designee to the U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado. Here he made a major contribution by lecturing to other officers on his experience and ideas as a part of the training program.

In 1955 he was appointed to the consultant advisory body of the newly organized Interdepartmental Committee on Nutrition for National Defense (ICNND). He participated in the initial planning and formulation of the committee's program directed to improve the nutritional health of the peoples of the developing countries. He was co-editor of the committee's "Manual for Nutrition Surveys," Interdepartmental Committee on Nutrition for National Defense, May, 1957. In January, 1956, he served as director of the first ICNND nutrition survey, which was conducted in Pakistan, and also served as deputy director of the Iran survey, conducted at the same time. Here again Cy's personal dedication and logical approach enabled him to receive utmost cooperation from specialists in a number of fields such as medicine, biochemistry, nutrition, food technology and agricultural economics, including Pakistani, Iranians and Americans. His sincerity and tact won him the admiration and friendship of all the team members and government officials with whom he came in contact. He never forgot his many friends in Pakistan and Iran and continued to follow their progress. In the following years he received repeated invitations to revisit both countries. Until his death, Cy continued in active participation with the ICNND, reviewing and advising on the various country survey reports and suggesting practical means of assisting these countries in utilizing their own food resources to their maximum ability.

His outstanding qualifications by this time were nationally and internationally known and, at the urgent request of world leaders in nutrition in 1957 he accepted the position of officer in charge of expanded aid to child and maternal nutrition with the United Nations Children's Fund (UNICEF). The acceptance of such an assignment involved a realization of its tremendous responsibility and the extreme difficulties which lay ahead, such as food habits, poverty of soil and people and even religion. Dedicated to a worthy cause, he accepted the challenge and resigned from the Pennsylvania State University January 1, 1958, to begin his new activities on a world-wide scope with an office at the United Nations Secretariat in New York. In selecting Dr.

French as first choice among the world's best known nutritionists, it was realized that he possessed the exceptional qualities upon which depended the success of the entire undertaking. It was necessary that he be not only scientifically sound in the field of nutrition but also that he be amiable and patient, pursuasive, and democratic in dealing with people of foreign lands. The best attributes of a diplomat and missionary were part of his stock-in-trade.

In the conduct of his new assignment, Cy traveled to many foreign countries, not as a mere visitor but to see and mingle with the common people, to learn their customs, their general mode of living and particularly their diets, to observe what crops were grown and to recommend the feasible introduction of new ones which could bring about a marked improvement in the native diet.

It was on an extended tour of Africa that he contracted amoebic infection of the liver from which he died on January 3, 1960, shortly after returning home.

The abrupt ending of a brilliant career was particularly tragic in that it came so early in life just as his greatest work was well underway. One can only surmise the extent of the loss to the countless undernourished peoples of the earth for whom he dedicated his life. May the inspiration of his accomplishments help those who follow in striving to provide adequate nutrition to all peoples of the earth.

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Effects of Saturated and Unsaturated Fats and Their Mixtures on the Lipid Metabolism of Monkeys[1]

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Saturated and unsaturated fats and their role in lipid metabolism have been the subject of innumerable investigations for almost a decade.

Kinsell and co-workers in 1952 reported that patients receiving a formula diet containing fat of vegetable origin had lower levels of serum cholesterol and phospholipids than patients fed a mixed diet or a formula diet containing butter fat or egg yolk fat. These observations have been repeatedly confirmed by Ahrens et al. ('54), Beveridge and co-workers ('55), Bronte-Stewart and associates ('56) and others.

Hegsted et al. ('57) have suggested that essential fatty acids (linoleic acid and perhaps arachidonic acid) act together with the saturated fatty acids in producing low serum cholesterol values in experiments in which rats were fed a diet low in protein (10% casein) and supplemented with 0.45% each of cholesterol and cholic acid. Investigators from the same laboratory (Portman et al., '56), using *Cebus* monkeys, fed isocaloric diets containing a fixed ratio of protein and cholesterol to calories but with different amounts of corn oil. Serum cholesterol and β-lipoproteins were higher when diets containing corn oil supplied 45 or 32% of the calories than with 10%. Hydrogenated cottonseed oil produced higher cholesterol and β-lipoprotein values at all levels than corresponding quantities of nonhydrogenated cottonseed oil. Hegsted et al. ('57) fed graded mixtures of tung oil high in oleostearic acid with low-protein diets containing 1% of cholic acid and 3% of cholesterol. When corn oil was admixed with tung oil, the lowering of serum cholesterol was in proportion to the amount of corn oil added. Keys and associates ('59) found that in man slightly more than 2 gm of linoleic acid counteracts the effect of 1 gm of saturated fatty acids in increasing serum cholesterol. Peifer and Holman ('59) reported that, with an adequate intake of essential fatty acids, increasing the saturated fat stimulated the growth rate of rats: with a deficiency of the essential fatty acids the saturated fats were not used for growth. It appears, therefore, that the essential fatty acids are necessary for proper utilization of the saturated fatty acids. Hegsted and coworkers ('59) recently devised a regression equation relating the amount of fatty acids (saturated, monounsaturated and polyunsaturated) in the diet to the serum cholesterol levels. The coefficient for the monounsaturated acid was positive and for the saturated and polyunsaturated acids negative. These authors suggest that the monounsaturated acid raises the serum cholesterol level, whereas the saturated and polyunsaturated acids reduce it, the saturated acid being about ¼th as active as the polyunsaturated acid. As pointed out, caution should be taken in attaching too much significance to the calculated co-efficient as only two rats were used in each group.

The study herein reported deals with the effects of graded levels of safflower oil or butter fat fed separately and combined upon plasma total lipids, cholesterol, total sterols, and phospholipids of young adult male rhesus monkeys maintained on purified diets.

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[1] This work was supported by a grant from the Nutrition Foundation, Inc., New York.

EXPERIMENTAL

Eleven male rhesus monkeys,[2] approximately 5 years of age, were segregated into groups and placed on experimental diets as indicated in table 1. Butter fat[3] and safflower oil[4,5] were fed separately and combined. The safflower oil used in this study contained 76% of linoleic acid and the butter fat, 4%. It was impossible to balance the intakes of linoleic acid and saturated fatty acids as both fats contained varying proportions of the two types of fatty acids. The butter fat and safflower oil were fed at the 20% level (groups 1 and 2) as extensive observations had been made in this laboratory, (Emerson et al., '60) with monkeys receiving this intake of these fats. The mixed fats were given at two levels: namely, (1) at 7.5% of butter fat to 13% of safflower oil which contained 10.1% of saturated and 10.4% of polyunsaturated fatty acids (group 3); and (2) 18% of butter fat to 1.8% of safflower oil which furnished 17.7% of saturated and 2.3% of polyunsaturated fatty acids (group 4). The monkeys were maintained with their respective diets for 6 months.

Daily food consumption and monthly weights were observed throughout the experimental period. The following determinations were made on plasma at monthly intervals: total lipids, free and total cholesterol, total sterols and phospholipids. Total lipids were extracted from plasma with dimethoxymethane and methanol in a ratio of 4 to 1. The free and total cholesterol was estimated by the Sperry-Webb ('50) modification of the original method of Schoenheimer-Sperry. Sterols and phospholipids were separated by the procedure of Fillerup and Mead ('53) using silicic acid chromatography.

RESULTS

General. The average daily food consumption was highest for the monkeys fed the diets rich in safflower oil (groups 2

[2] The monkeys used in this study were a gift from Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

[3] Supplied by the Knudsen Creamery Company, Los Angeles, California.

[4] Courtesy of Pacific Vegetable Oil Corporation, Richmond, California.

[5] The vitamins other than biotin were supplied by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey. The biotin was furnished by Hoffmann-LaRoche, Inc., Nutley, New Jersey.

TABLE 1

Composition of diets

	Groups			
	1 (2)[1]	2 (2)	3 (3)	4 (4)
	gm/100 gm		*gm/100 gm*	
Butter fat	20.0	—	7.5	18.0
Safflower oil	—	20.0	13.0	1.8
Salts (Hegsted)	4.0	4.0	4.0	4.0
Casein	24.0	24.0	24.0	24.0
Sucrose	52.0	52.0	51.5	52.2
Saturated fatty acids	19.5	4.4	10.1	17.7
Polyunsaturated fatty acids	0.8	15.6	10.4	2.3

Addendum		Fed sugar cubes daily	
Inositol	0.04		
p-Aminobenzoic acid	0.04		*Mg*
Choline·Cl	0.10	Thiamine·HCl	1
α-Tocopherol	0.015	Riboflavin	1
Vitamin A and D (containing 1500 units A, 150 units D)	0.003	Ca pantothenate	3
		Niacinamide	5
		Pyridoxine·HCl	2
		Vitamin K (menadione)	1
		Ascorbic acid	25
			μg
		Biotin	20
		Folic acid	500
		Vitamin B_{12}	25

[1] Figures in parentheses indicate animals/group.

and 3). The animals in all groups maintained their weights throughout the experimental period. The weight changes did not correspond to the food intakes as the animals receiving 20% of butter fat (group 1) gained 0.31 kg over the 6-month period with a daily intake of 217 gm of food, whereas the monkeys receiving 7.5% of butter fat and 13% of safflower oil, (group 3) gained only 0.09 kg with an average daily food intake of 286 gm (table 2). It is difficult, however, to make generalizations from small groups. All monkeys were in excellent clinical condition throughout the test period. (Monkeys fed purified diets of high fat content have loose feces.)

Biochemical findings. The total plasma lipids were, as expected, highest for the monkeys receiving the diet containing 20% of butter fat, (group 1) averaging 904 mg per 100 ml and lowest for the groups fed 7.5% of butter fat with 13% of safflower oil (group 3) and 20% of safflower oil alone (group 2) averaging 557 and 606 mg per 100 ml, respectively. The value was intermediate for the monkeys receiving 18% of butter fat with 1.8% of safflower oil (group 4), namely 720 mg per 100 ml (fig. 1).

Determinations of free and total plasma cholesterol (fig. 2) were made at monthly intervals. The values followed the same pattern as those of the total lipids ranging from a high of 224 mg per 100 ml (total) for the monkeys fed 20% of butter fat (group 1) to a low of 157 mg per 100 ml for the groups receiving the mixture of 7.5% of butter fat with 13% of safflower oil (group 3) and 20% of safflower oil alone (group 2). An intermediate figure (170 mg per 100 ml) was observed with

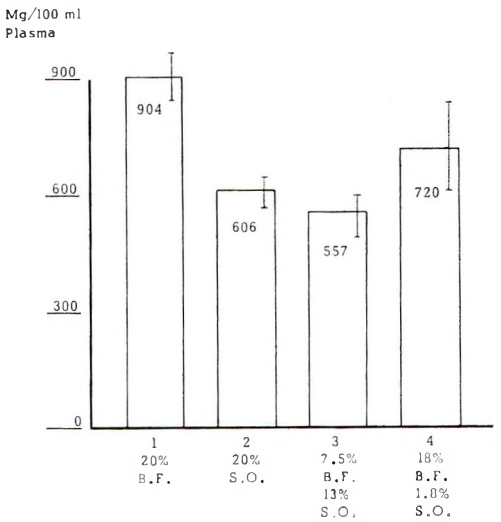

Fig. 1 Total plasma lipids, average of 6 monthly determinations; I indicates range of averages for individual monkeys.

18% of butter fat and 1.8% of safflower oil (group 4).

The total sterol esters fractionated by silicic acid chromatography were, as expected, slightly higher than could be accounted for by cholesterol alone. Again the same pattern prevailed (fig. 3). The phospholipids constituted almost 50% of the total lipids from the plasma of the monkeys on each of the dietary regimens. The phospholipids also followed the pattern described for total lipids, cholesterol and total sterols (fig. 4).

DISCUSSION

Safflower oil containing 76% of linoleic acid, when admixed with butter fat produced a lowering of total plasma lipids, total sterols, including total and free cho-

TABLE 2

Food intake and weights

Group	Diet	Av. daily food consumption	Initial weight	Av. weight gain
		gm	*kg*	*kg*
1	20% Butter fat	217	5.23	0.31
2	20% Safflower oil	325	4.95	0.33
3	7.5% Butter fat 13% Safflower oil	286	6.52	0.09
4	18% Butter fat 1.8% Safflower oil	216	6.59	0.12

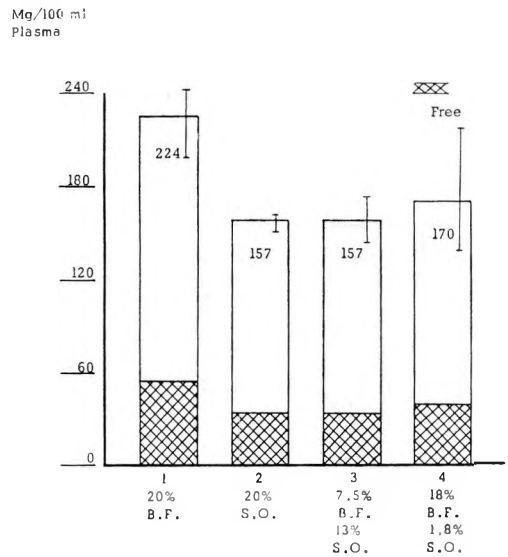

Fig. 2 Free and total plasma cholesterol, average of 6 monthly determinations; I indicates range of averages for individual monkeys.

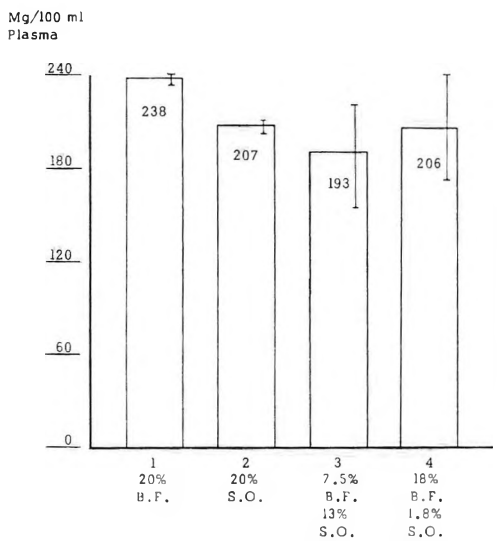

Fig. 3 Sterol esters, average of 6 monthly determinations; I indicates range of averages for individual monkeys.

lesterol and phospholipids, when fed to young adult male rhesus monkeys.

The values for all lipid fractions were as low when a mixture of approximately 7.5% of butter fat and 13% of safflower oil (representing a 1 to 1 ratio of saturated to polyunsaturated fatty acids) was

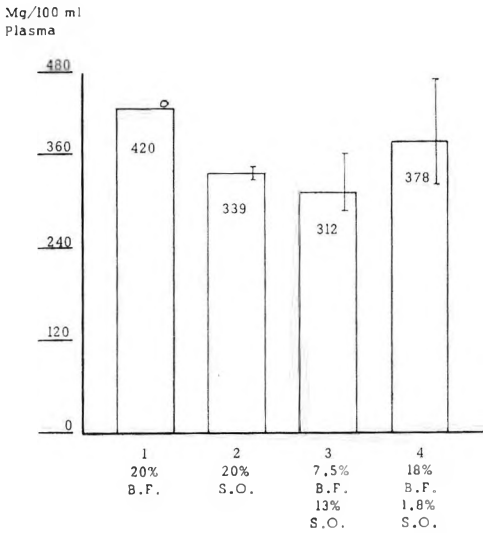

Fig. 4 Phospholipids, average of 6 monthly determinations; I indicates range of averages for individual monkeys.

fed as with 20% of safflower oil. The total lipids and fractions contained therein were lowered when 1.8% of safflower oil was given with 18% of butter fat, (ratio of 8 to 1 of saturated to polyunsaturated fatty acids). One of the 4 animals in this group had higher lipid values in each of the 6 determinations than the other monkeys in the group. The rhesus monkey would appear to be sensitive to a low level of unsaturated fat combined with a high level of saturated fat in the lowering of plasma cholesterol. The magnitude of the effect was greater than that observed by Hegsted and co-workers, ('57) with rats fed diets low in protein and containing mixtures of tung oil and corn oil and supplemented with cholesterol and cholic acid. When 10% of tung and 10% of corn oil were fed, the serum cholesterol values were almost three times as high as with 20% of corn oil alone. With 20% tung oil alone the value was 4.5 times that for 20% of corn oil. Keys et al. ('59) reported that slightly more than 2 gm of linoleic acid counteracted 1 gm of saturated fatty acids in man. The monkey would, therefore, appear to be more sensitive than man to the effect of polyunsaturated fatty acids upon plasma lipids.

SUMMARY

Young, adult male rhesus monkeys about 5 years of age were maintained for 6 months with purified diets containing 20% of butter fat, 20% of safflower oil or mixtures of these fats. The mixed fats were fed on a basis of approximately 10% of saturated and 10% of polyunsaturated fatty acids and 18% of saturated and 2% of polyunsaturated fatty acids, respectively. The animals were maintained in good condition and all groups made slight weight gains. Plasma was examined for total lipids, free and total cholesterol, total sterols, and phospholipids. Total lipids and their fractions were highest for the group fed 20% of butter fat in the diet. Average values for plasma lipid constituents were lowered by the feeding of as little as 1.8% of safflower oil to 18% of butter fat (2.3% of polyunsaturated to 17.7% of saturated fatty acids): a maximal lowering was observed when the mixture containing 13% of safflower oil and 7.5% of butter fat (10.1% of polyunsaturated to 10.4% of saturated fatty acids) was fed. The levels with the latter were as low as observed when safflower oil was fed as the sole fat at the 20% level.

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The Free Blood Plasma Amino Acids of Swine as Related to the Source of Dietary Proteins[1]

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It has been known for several years that the nutritive value of a dietary protein is mainly determined by its amino acid content. Not all amino acids present in a protein, however, are available to the animal, especially when the protein is either from a vegetable source or has had some of the amino acids destroyed or rendered unavailable by processing.

Methionine balance studies by Melnick et al. ('46) showed that 49% of the methionine present in soybean meal fed to rats appeared in the feces and therefore was not available to the animal. Kuiken ('52) reported variations in availability of lysine and methionine in cottonseed meal, depending on the conditions of processing.

Whether this reduced availability is due to destruction of the amino acids caused by the conditions of processing or due to their particular linkage in the protein, it would appear that a measure of such availability could be determined by the extent to which they appear into the blood stream, being thus rendered available to the animal at the tissue level.

Richardson et al. ('53) reported that plasma amino acids in the chick did not parallel the amounts fed in the diet. Some of the amino acids present in low amounts showed an unexpectedly high level in the blood. These same authors, however, as well as Charkey et al. ('53) and Denton et al. ('53), presented evidence that the concentration of any one amino acid in the blood is usually in agreement with the relative concentration of that amino acid in the diet; and that the addition of supplemental amino acids to the diet results in an increase in the blood level of the corresponding amino acid. Also, a lowering of the concentration of any one amino acid in the blood has been observed by these authors to be the reflection of a deficiency of this particular amino acid in the diet.

More recently, Longenecker and Hause ('59) reported that the free amino acid level in the plasma of the dog reflects the composition of the diet. They described a procedure by which the amino acid adequacy of a diet can be evaluated through the study of the plasma free amino acids of an animal fed the protein being assayed.

The present study was conducted to establish the difference in blood plasma free amino acids of young swine fed different sources of protein; also to obtain information concerning the availability of some of the essential amino acids when provided by different proteins as compared to milk proteins, which are regarded as giving optimal performance and most satisfactory growth response in this species.

EXPERIMENTAL

Forty crossbred pigs averaging 11.4 pounds of body weight and 22 days of age were selected and randomly assigned by weight within litter to a randomized block design. Two pens of 4 pigs per pen were subjected to each of the 5 treatments.

Feed and water were consumed ad libitum. Weight gains and feed efficiency were determined weekly and the experiment was terminated after 28 days. The diets used contained by weight (in per cent): ground yellow corn, 25; lactose, 25; stabilized lard, 2; calcium carbonate, 1.5;

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[1] Journal paper no. J-4069 of the Iowa Agricultural and Home Economics Experiment Station, Ames. Project no. 959.

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dicalcium phosphate, 4.0; trace mineral mixture,[4] 0.2, iodized salt, 0.5; and vitamin premix,[5] 0.3, plus the corresponding amount of the protein source and corn starch to provide a 20% protein diet. The 5 sources of protein[6] used were: dried skim milk (DSM), soybean meal (SBM), fish meal (FM), meat meal (MM) and cottonseed meal (CSM).

The blood specimens were collected after the experimental diets had been fed for 26 days, without any previous fasting. Six milliliters of blood were collected in heparinized tubes from the anterior vena cava of each of the 4 pigs in a pen and the 4 samples pooled. The plasma was obtained immediately by centrifugation at 2,500 rpm. for 10 minutes, the plasma proteins were removed by treatment with picric acid and the samples prepared for column chroma-

[4] Contributed to diet, in ppm: Fe, 141; Cu, 10; Co, 3; Zn, 163; and Mn, 114.

[5] Contributed to diet, per pound: vitamin A, IU, 3,000; vitamin D_2, IU, 1,000; riboflavin, 4.4 mg; Ca pantothenate, 7.0 mg; niacin, 24.0 mg; choline, 227.0 mg; vitamin B_{12}, 20.0 μg; and folic acid, 500.0 μg.

[6] Protein content, per cent: DSM, 31.2; SBM, 50.4; FM (menhaden), 57.2; MM, 61.2 and CSM, 41.0.

TABLE 1

Free amino acid content of plasma[1] and amino acid content of complete diets[2] in pigs fed varying sources of protein

Amino acid	Dietary protein				
	DSM[3]	SBM[3]	FM[3]	CSM[3]	MM[3]
	mg/100 ml plasma	*mg/100 ml plasma*	*mg/100 ml plasma*	*mg/100 ml plasma*	*mg/100 ml plasma*
Taurine	0.97	0.39	0.53	0.84	0.58
Aspartic acid	0.41	0.26	—[4]	0.14	—[4]
Threonine	3.38	2.10	3.10	2.02	2.36
Serine[5]	3.02	3.74	2.88	3.34	4.46
Glutamic acid	3.34	2.20	3.28	1.90	1.72
Citrulline	0.98	1.46	1.55	1.06	1.38
Proline	9.50	3.10	3.25	2.92	4.00
Glycine	4.62	5.40	12.32	5.14	12.84
Alanine	5.56	4.17	5.36	4.24	3.02
Cystine	1.18	0.51	0.90	0.22	0.29
Valine	4.93	2.81	3.54	2.39	2.15
Methionine	1.13	—[6]	0.84	0.30	0.45
Isoleucine	2.12	1.72	1.76	0.89	0.87
Leucine	3.88	2.08	2.30	1.20	1.69
Tyrosine	3.32	1.68	1.10	1.26	0.99
Phenylalanine	1.52	1.02	0.81	1.72	1.47
Lysine[7]	7.44	5.33	5.06	3.58	3.64
Histidine	1.75	1.38	1.82	1.38	1.55
Arginine	2.55	2.13	3.27	4.72	2.02
	% diet	*% diet*	*% diet*	*% diet*	*% diet*
Threonine	0.95	0.72	0.99	0.62	0.56
Glutamic acid	4.60	3.62	—[8]	3.45	3.06
Glycine	0.22	1.03	1.35	1.15	2.09
Cystine	0.29	0.27	0.24	0.45	0.18
Valine	1.39	0.98	1.23	0.93	0.80
Methionine	0.52	0.30	0.60	0.30	0.25
Isoleucine	1.30	0.96	1.37	0.76	0.57
Leucine	2.09	1.46	1.79	1.25	1.16
Tyrosine	1.01	0.67	0.72	0.64	0.36
Phenylalanine	1.03	0.88	0.95	0.98	0.62
Lysine	1.55	1.09	1.72	0.79	0.96
Histidine	0.53	0.45	0.55	0.48	0.31
Arginine	0.78	1.23	1.35	1.64	1.14

[1] Average values of two replications, one in MM, each replication being a pooled sample of 4 pigs on the same treatment.

[2] According to literature values (Lyman et al., '56, '58; Hubbell, '60).

[3] DSM indicates dried skim milk; SBM, soybean meal; FM, fish meal; CSM, cottonseed meal; and MM, meat meal.

[4] Less than 0.1 mg.

[5] Not corrected for asparagine nor glutamine.

[6] Less than 0.15 mg.

[7] Not corrected for ornithine.

[8] Value unKnown.

tography as described by Stein and Moore ('54). At this point the samples were stored at − 10°C in polyethylene bottles until analyzed.

The concentration of amino acids in the protein-free plasma was determined by ion exchange chromatography as described by Moore et al. ('58). Cysteine was oxidized to cystine prior to analysis. A correction for a 10% loss of methionine on the column was made and all amino acids were corrected for color yield using factors given by Moore and Stein ('54). Tryptophan was not determined in any of the samples.

RESULTS

Plasma amino acid concentrations are presented in table 1. Due to overlapping between serine, asparagine and glutamine in the effluent curve, the values for serine include these two amines, although it is believed that the quantity of these present in the plasma is very low. Also, the values for lysine include ornithine, since the methods used did not separate the two, both amino acids emerging in a common peak.

Growth and feed efficiency data are shown in figure 1. The plasma values for urea are presented in the same figure as they appear to be inversely proportional to the gain in body weight and feed efficiency, which would be expected on the basis of the amino acids from the unbalanced proteins being catabolized and excreted via urea. The amino acid composition of the 5 different diets, based on literature values and expressed on a percentage basis, is presented in table 1.

In figure 2 the plasma amino acids from the animals fed the various diets are compared with the amounts present in the respective diet. Note, for the pigs fed SBM, the low values for cystine, phenylalanine, tyrosine, isoleucine and leucine. The values for methionine were so low that they escaped detection by the method of analysis used.

When the plasma amino acid concentrations from the animals fed the DSM diet are graphically presented, a much higher value for most amino acids is evident, especially for the two sulfur-containing amino acids, cystine and methionine. The concentration of phenylalanine, 1.52 mg

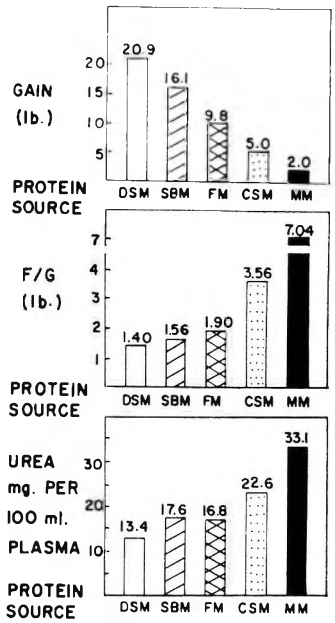

Fig. 1 Growth, feed efficiency and blood plasma urea values of young pigs fed different sources of proteins. DSM indicates dried skim milk; SBM, soybean meal; FM, fish meal; CSM, cottonseed meal and MM, meat meal. Error mean square for testing treatment effects equals: 7.70 for total gain; 2.59 for F/G.

per 100 ml, is low but far above the value found in the case of the SBM-fed animals, 1.02 mg. Also a high concentration of glycine was observed in the blood of these animals, considering the low level present in the diet and apparently indicating an active process of synthesis.

Practically the same amino acids appearing in the lowest concentration in the blood of the pigs consuming the SBM diet, show also the lowest concentration in the case of the animals fed the FM diet. Although present in higher concentration than in the blood of the animals fed the SBM diet, methionine is still low as compared with the level found in the plasma of the DSM-fed pigs. Along with low concentrations of methionine, isoleucine, tyrosine and leucine the lowest value for phenylalanine was obtained, 0.81 mg per 100 ml. The concentration of glycine was very high, 12.3 mg in the individuals fed the FM diet, which is in agreement with the high content of this particular amino acid in this protein source.

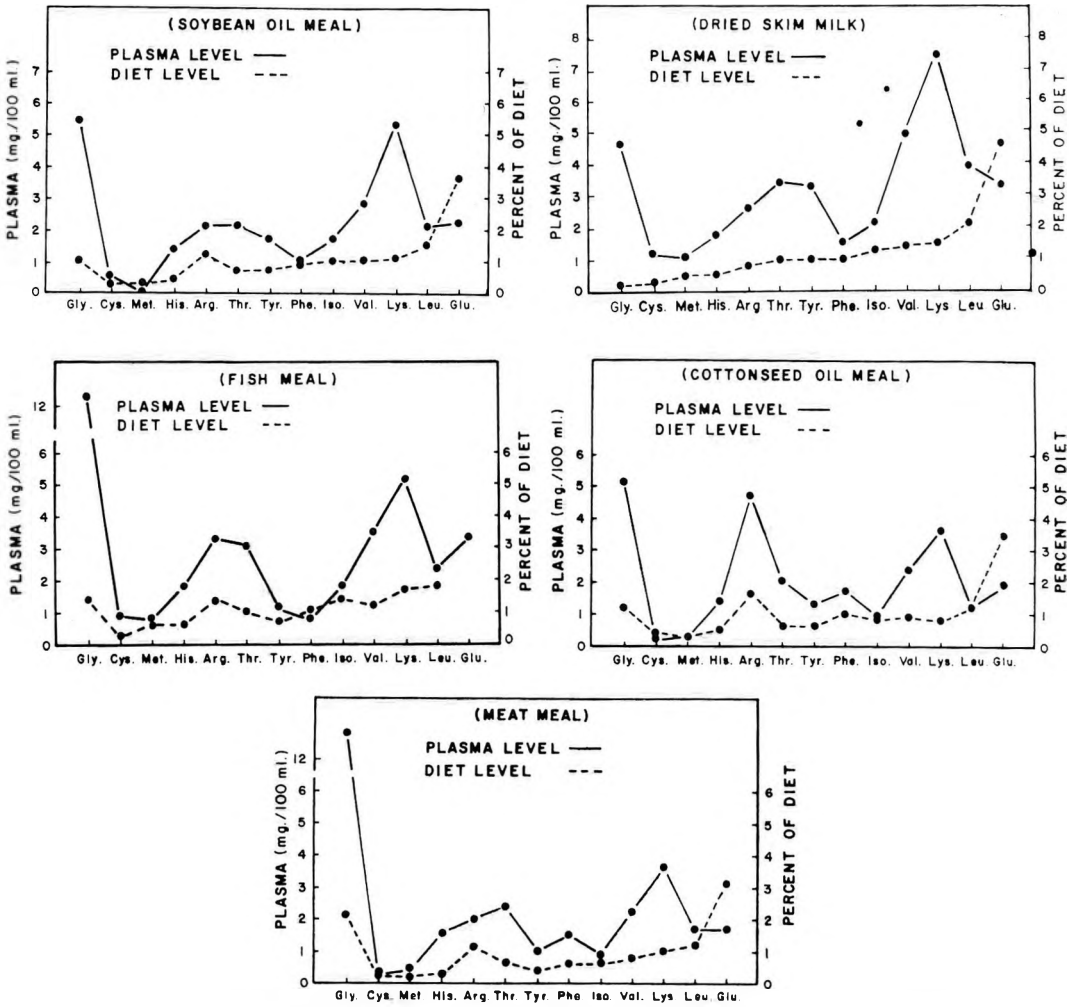

Fig. 2 Blood plasma aminograms and amino acid content of the 5 experimental diets.

A concentration of 1.72 mg of phenylalanine per 100 ml, in the animals fed CSM, was the highest plasma level for this amino acid throughout the 5 different treatments, despite the fact that the content of phenylalanine in the CSM diet is lower than that in the DSM diet (0.98 and 1.03% respectively) indicating a higher availability of this amino acid in cottonseed oil meal. The values for tyrosine, however, did not follow the same trend since the plasma concentration of tyrosine appears to be much higher, (3.32 mg per 100 ml) in the blood of the pigs fed the DSM ration than in the case of the CSM diet, (1.26 mg per 100 ml), which is in close agreement with the levels present in the respective diets. Both cystine and methionine were consistently low, 0.22 mg and 0.30 mg per 100 ml, respectively, as were isoleucine, 0.89 mg and leucine, 1.20 mg per 100 ml.

The lowest concentrations for practically all the amino acids, with the exception of glycine, were noted in the plasma of the animals fed the MM diet. This diet also gave the poorest growth response and feed efficiency, as compared with the other 4 diets. This poor performance with the MM diet may be largely a reflection of tryptophan deficiency that was not measured in these studies. A very high concentration

of glycine, 12.8 mg per 100 ml, accompanied very low values for cystine, methionine, leucine. and the lowest value obtained for isoleucine, 0.87 mg per 100 ml of plasma.

A more direct comparison of the amino acids leucine, phenylalanine, methionine, histidine, threonine and arginine among the 5 different diets is shown in figure 3. Some amino acids appeared in the plasma in a uniform way which seems to be predictable, regardless of the nature of the protein providing them, namely, histidine and threonine, whereas in the case of other amino acids, namely methionine and phenylalanine, plasma concentration seems to be determined more by the nature of its dietary carrier than the actual amount present in the protein.

DISCUSSION

These studies indicate that the plasma concentration of individual amino acids depends to a certain degree upon the amount present in the diet, with the exception of some of the nonessential amino acids, such as glycine and glutamic acid. In the comparison among the 5 different sources of protein, which gave a totally different growth response when fed to baby pigs, the concentration of free amino acids in the blood plasma seems to be related not only to the amounts present in the protein ingested but also to the nature of this protein. Also, a higher and more relatively uniform concentration of free amino acids was observed in the blood plasma of the animals growing at a faster rate. The relative proportion of these amino acids seemed to be more important than the total concentration, when plasma amino acid patterns and growth response of the animal were compared. Most of the plasma amino acids in the pigs fed soybean meal, cottonseed meal, meat meal and fish meal are maintained at a much lower concentration than those in the dried skim milk, which may indicate either a higher utilization at the tissue level or a poor absorption from the gastrointestinal tract.

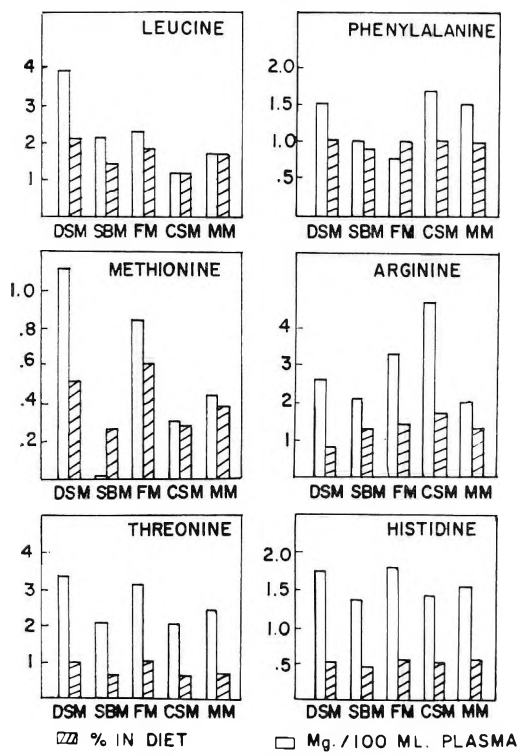

Fig. 3 Blood plasma free leucine, phenylalanine, methionine, arginine, threonine and histidine versus the dietary content of these amino acids in pigs fed 5 different protein sources.

In view of the several factors which interact in the blood amino acid picture (amino acid withdrawal from the blood by the tissues, amino acid catabolism in the liver, degree of absorption at the intestinal wall, etc.) it is difficult to assign a definite significance to these different blood plasma levels as they relate to the amino acid make-up of the dietary proteins. It is to be expected, however, that the variation in the concentration of the essential amino acids in the plasma, as compared with the amounts provided by the diet, may be partially due to a fast removal from the blood stream by the tissues, although these differences reflect more likely a difference in the degree of availability at the level of the digestive tract. It is therefore evident that not all the amino acids present in the diet are completely absorbed, this unequal absorption producing the breaking points in the blood amino acid pattern as compared with the "standard" pattern noted in the animals consuming milk diets. An increased catabolism of the excess amino acids in some of these diets, as indicated by increased blood urea values, would explain the poor performance obtained with

these diets as measured by growth and feed efficiency.

SUMMARY

Plasma amino acid levels in the young pig have been determined and found to be related to the amino acid composition of the dietary protein. Different plasma amino acid patterns have been observed for the 5 different protein sources analyzed, dried skim milk, soybean meal, fish meal, cottonseed meal and meat meal. These plasma amino acid patterns indicate, by comparison with the concentrations observed in the animals fed milk proteins, some of the amino acids that may be responsible for the inability of the young pig to maintain an optimal rate of body gain when fed a ration with some of these proteins as the source of supplementary protein.

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The Mineral Requirements of the Dog[1]

III. THE MAGNESIUM REQUIREMENT

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Magnesium has been shown to be an essential element and its requirement has been studied in the rat (Tufts and Greenberg, '38; Kunkel and Pearson, '48a; Hegsted et al., '56; McAleese and Forbes, '61), pig (Mayo et al., '59; Bartley et al., '61), guinea pig (O'Dell et al., '60), rabbit (Kunkel and Pearson, '48b), calf (Blaxter et al., '54), chick (Almquist, '42), and duck (Van Reen and Pearson, '53) but not in the dog. Among the various species, marked differences as well as similarities have been noted in the pathology of magnesium deficiency (O'Dell, '60), but again only a little information (Orent et al., '32) is available concerning the dog. It therefore seemed worthwhile both to establish the minimum dietary requirement of magnesium for the weanling dog and to describe the pathological changes which accompany magnesium insufficiency in this species.

EXPERIMENTAL

The composition of the low-magnesium basal ration (no. 58-M) formulated by us for use in this study appears in table 1. It contained by analysis 80 ppm of magnesium on a dry-weight basis. Magnesium supplements were added as anhydrous magnesium sulfate.

Jenkins[3] in preliminary studies observed both poor growth and convulsions in weanling mongrel pups fed diet 58-M. Equal and normal growth and development occurred, however, in pups fed diet 58-M supplemented with either 100, 200, 400, or 1,000 ppm of magnesium. Balance studies indicated that absorption of magnesium was excellent from this diet and that 45 to 50% of the magnesium was retained when the dietary content was either 80 or 180 ppm. Using these data as an indication of the probable range of adequacy of magnesium, two experiments were performed.

TABLE 1

Composition of low-magnesium basal ration, 58-M

	%
Casein, extracted[1]	21.0
Sucrose	66.1
White grease, stabilized[2]	8.0
Salts[3]	4.8
Vitamins[4]	0.1

[1] Extracted with hot ethanol.
[2] Product of Oscar Mayer and Company, Madison, Wisconsin.
[3] For 100 gm of ration: (in grams) KCl, 1.14; iodized NaCl, 1.00; $CaCO_3$, 1.50; Na_2HPO_4, anhydrous, 1.15; and (in milligrams) $Fe_2(SO_4)_3$, 36.0; $CuSO_4 \cdot 5H_2O$, 2.15; $MnSO_4 \cdot 1H_2O$, 1.54; $ZnCl_2$ (dry), 0.92; and $CaCl_2 \cdot 6H_2O$, 0.88.
[4] For 100 gm of ration: (in milligrams) thiamine·HCl, 0.066; riboflavin, 0.176; nicotinamide, 0.900; Ca pantothenate, 0.200; pyridoxal·HCl, 0.088; folic acid, 0.030; *d*-biotin, 0.010; also vitamin B_{12}, 2.2 μg and choline chloride, 0.123 gm; *dl*-α-tocopherol was added to halibut liver oil, and the mixture administered every 3 to 4 days, providing 1 mg of tocopherol, 10 to 15 IU of vitamin A/pound of body weight/day.

In the first experiment, 4 litters of weanling Beagle pups were distributed among three dietary lots so as to provide 4 pups per lot. The basal ration 58-M (80 ppm of magnesium) was fed to lot 1. The control ration which contained an additional 100 ppm of magnesium was fed to lot 2. Another control ration[4] composed of natural food products, providing by analysis 1,300 ppm of magnesium on a dry-weight basis, was fed the pups in lot 3. Weight changes and the presence or absence of typical mag-

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[2] Present address: U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver 30, Colorado.
[3] Jenkins, K. J. 1958 A study of some mineral requirements of the dog. Ph. D. Thesis, University of Wisconsin.
[4] This ration was formulated by Dr. Zoe Anderson, Director of Nutrition Research for the National Dairy Council. It consisted of meat, dairy products, vegetable, fruit and cereal foods in the proportion usually recommended for human consumption. The contents of this ration were provided by the National Dairy Council and were mixed and packaged by Albers Milling Company, Jefferson, Wisconsin.

nesium deficiency symptoms were recorded for 14 weeks.

Since the results of the preliminary work (Jenkins, '58) and the first experiment indicated that the response of the Beagles and similar-size mongrels was comparable, both were used in the second experiment. In the latter, 5 litters of weanling pups (two of mongrels, three of Beagles) were each distributed among 5 lots and fed diet 58-M with the following variations in magnesium content: lot 1, 80 ppm; lot 2, 100 ppm; lot 3, 120 ppm; lot 4, 140 ppm; and lot 5, 180 ppm. Each lot had 4 pups except lot 5 which contained only three. The pups from 4 of the 5 litters were sacrificed after 7 to 8 weeks on experiment but the 5th litter (three pups, one each from lots 3, 4, and 5) was allowed to consume the diet for 10 weeks.

The Beagle pups were from our own kennels, and the mongrels were purchased locally. The latter were immediately treated to control parasites. As a precaution against distemper, infectious hepatitis, and *Leptospira canicola* infections, immune serum was administered to all experimental animals from 6 until 12 weeks of age at which time they were vaccinated against these diseases.

At the termination of each experiment blood samples were collected and allowed to clot. Serum was analyzed for magnesium, calcium, and inorganic phosphorus. Following the postmortem inspection, the thoracic aorta was removed and analyzed for total ash, calcium, phosphorus and magnesium.

ANALYTICAL METHODS

The aorta samples were dried, heated for 18 hours at 600°C, and the resultant alkaline ash was dissolved in 0.6 N HCl. The method of Robinson and Rathbun ('59) involving titration with EDTA in a buffered solution of Eriochrome Black T was used for determination of the calcium and magnesium in aliquots of the aorta ash and in blood serum. Serum inorganic phosphorus and aortic total phosphorus were measured by the Fiske-SubbaRow method as outlined by Hawk et al. ('54).

RESULTS AND DISCUSSION

A characteristic syndrome of magnesium deficiency appeared in both the first and second experiments when the level of dietary magnesium was below 140 ppm. The severity of the syndrome appeared to be reduced, however, in lots 2 (100 ppm) and 3 (120 ppm) in the second experiment. Anorexia developed in the pups fed the low-magnesium rations (lot 1, experiment 1; lots 1, 2, 3, experiment 2) after two to three weeks on experiment. The anorexia was accompanied by a general decline in weight gain as compared with that in the control groups. The average cumulated weight gains per week for the first experiment are presented in table 2. By the 5th week, muscular weakness was evident. Exercise was generally followed by prolonged periods of inactivity and hyperirritability in contrast with the reaction of vigor and good muscle tone in the control pups. Continued muscular weakness and atrophy resulted in a relaxation of the

TABLE 2

Selected representative periodic weight gain data of the growing dog fed a low-magnesium diet

	Average cumulated gain[1]		
	kg	*kg*	*kg*
Magnesium content, ppm	80	180	1300
No. of animals/lot	4	4	4
Av. initial weight, kg	2.75 ± 0.09[1]	2.93 ± 0.21[1]	2.64 ± 0.26[1]
Weeks on experiment			
2	0.89 ± 0.21	1.15 ± 0.19	0.70 ± 0.11
5	1.33 ± 0.10	2.65 ± 0.22	1.95 ± 0.23
10	1.55 ± 0.18	3.80 ± 0.20	3.88 ± 0.41
14	1.31 ± 0.15	4.07 ± 0.11	4.53 ± 0.54

[1] Mean ± standard error of the mean.

Fig. 1 Left, typical phalanges relaxation in a growing dog fed a low-magnesium ration containing 80 ppm of magnesium (12 weeks on experiment). Right, basal diet + magnesium to supply 180 ppm of magnesium.

muscles and tendons in the legs, particularly noticeable in the area of the carpus and the phalanges (fig. 1). The hindquarters were also affected and showed evidence of ataxia. One or more convulsive attacks were observed in the majority of the deficient pups.

Mineral deposition was easily visible upon inspection of the lumenal wall of the thoracic aorta at necropsy (fig. 2). These lesions were observed in all of the lot 1 (80 ppm of magnesium) animals in experiment 1; in 7 of 8 pups in lots 1 and 2 (80 and 100 ppm), experiment 2; and in two of 4 pups in lot 3 (120 ppm), experiment 3. No such lesions were noted in the pups from the remaining lots. The deposits formed clearly defined thickened grayish-white areas, the surfaces of which appeared roughened. Upon microscopic section, they were observed to occur in both the intimal and medial layers in association with hyaline degeneration and inflammatory necrotic changes. These deposits were observed over a considerable

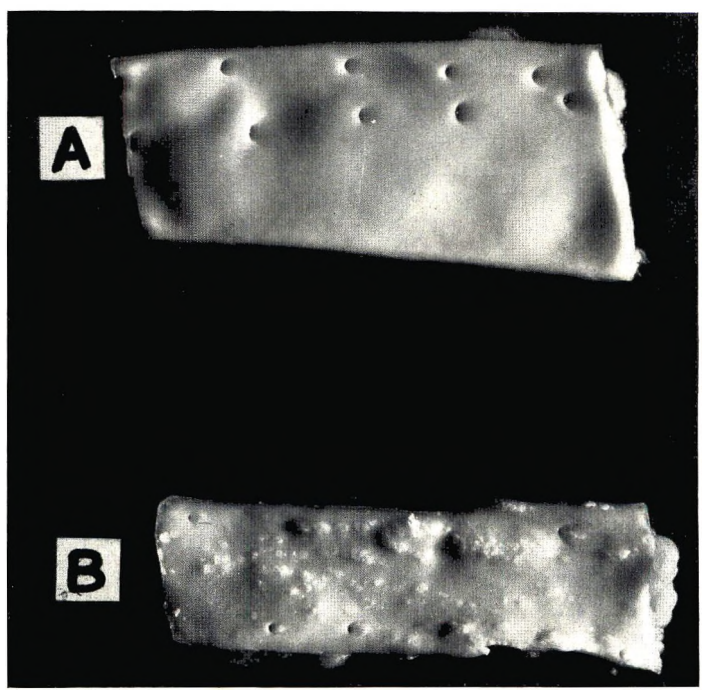

Fig. 2 (a) Normal thoracic aorta, obtained from a Beagle pup fed 180 ppm of magnesium. (b) The thoracic aorta from a litter mate of the dog above fed the basal ration containing 80 ppm of magnesium.

area of the thoracic aorta but were mainly concentrated in the regions just anterior to the openings of the small subsidiary vessels. Although the thoracic aorta appeared to be a major site of this lesion, subsequent examinations revealed similar deposits in the carotid artery, trachea, kidney, and spleen. No such visible lesions were noted in the cardiac or skeletal muscle or in the liver.

Aortic analysis (table 3) revealed an average threefold elevation in total ash, a forty-fold elevation in calcium, and a fourfold elevation in phosphorus in the group fed the low magnesium in experiment 1 as compared with either of the positive control groups. No appreciable change, however, could be detected in the content of total magnesium. The large increase in calcium and phosphorus as well as ash supports the concept that the mineralization was essentially calcification and that the primary anion was phosphate. The average difference in milligrams of calcium and phosphorus per 100 grams of dry tissue between the deficient and the control animals was 1,685 and 775, respectively, approximately the same 2:1 ratio noted in the common calcium phosphate salts. Qualitative tests (Feigl, '46) were negative for the presence of oxalate but were positive for the presence of carbonate. In experiment 2, the average aorta calcium levels (table 3) were elevated about tenfold when only 80 to 100 ppm of magnesium were present in the ration and eightfold at 120 ppm. When the dietary magnesium was either 140 or 180 ppm, however, the aortic calcium values were equal to those of the two control lots from experiment 1. Aorta total ash and phosphorus values showed a similar trend and again no variation in the average aorta magnesium content as a function of the level of dietary magnesium could be detected. This is in agreement with the result of MacIntyre and Davidson ('58) and McAleese and Forbes ('61) both of whom reported a slight decline in muscle magnesium from deficient animals but noted no such loss from calcified soft tissue. The greater accumulation of aortic mineral in the animals from lot 1, experiment 1, as compared with those from lot 1, experiment 2 was presumably a result of the more prolonged

TABLE 3

Effect of magnesium intake upon aortic ash calcium, phosphorus and magnesium content

	Experiment 1[1]			Experiment 2[2]				
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
Diet Mg, ppm	80	180	1300	80	100	120	140	180
No. of animals	4	4	4	4	4	4	4	3
No. of animals with aortic lesions	4	0	0	3	4	2	0	0
Average % ash, dry weight[3]	7.55 ± 0.63	2.59 ± 0.20	3.37 ± 0.29	3.35 ± 0.77	3.64 ± 0.23	3.20 ± 0.72	2.35 ± 0.17	2.35 ± 0.26
Average Ca, mg/100 gm dry tissue[3]	1725 ± 359	40 ± 6	54 ± 6	558 ± 305	567 ± 142	420 ± 317	54 ± 17	72 ± 16
Average P, mg/100 gm dry tissue[3]	1107 ± 111	332 ± 35	369 ± 26	600 ± 168	561 ± 45	512 ± 163	376 ± 26	351 ± 6
Average Mg, mg/100 gm dry tissue[3]	45 ± 5	34 ± 4	32 ± 3	46 ± 1	42 ± 3	60 ± 7	48 ± 4	45 ± 11

[1] Fed diets for 14 weeks.
[2] Fed diets for 7, 8, or 10 weeks.
[3] Mean ± standard error.

TABLE 4

Effect of magnesium intake upon blood serum magnesium, calcium, and phosphorus levels

	Experiment 1[1]			Experiment 2[2]				
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
Dietary Mg, ppm	80	180	1300	80	100	120	140	180
No. of animals	4	4	4	4	4	4	4	3
	Average concentration in mg/100 ml serum[3]							
Mg	0.4 ± 0.1	2.1 ± 0.3	2.0 ± 0.2	1.0 ± 0.4	0.9 ± 0.3	1.8 ± 0.4	1.6 ± 0.3	1.5 ± 0.1
Ca	9.5 ± 0.5	11.5 ± 0.6	12.4 ± 0.3	8.6 ± 1.6	9.1 ± 0.8	9.5 ± 1.1	11.2 ± 0.8	11.3 ± 0.4
P	8.0 ± 0.9	8.5 ± 0.3	8.0 ± 0.4	9.8 ± 0.9	9.7 ± 0.8	7.9 ± 1.0	7.5 ± 0.6	7.3 ± 0.2

[1] Fed diets for 14 weeks.
[2] Fed diets for 7, 8, or 10 weeks.
[3] Mean ± standard error.

intake of the low-magnesium diet by the former.

The average serum magnesium (table 4) was decreased to about 1.0 mg per 100 ml or less when the dietary magnesium level was below 120 ppm. At 120 ppm or higher, however, the average serum magnesium values were between 1.5 and 2.1 mg per 100 ml. A decline in the serum calcium and an elevation in the serum inorganic phosphorus was observed to accompany the depression of serum magnesium. This trend was of interest since species variation and conflicting results have been reported previously concerning the behavior of these substances during magnesium deficiency (Kruse et al., '32; Orent et al., '32; Duncan et al., '35; Blaxter et al., '54; MacIntyre and Davidson, '58; Maynard et al., '58). It appeared desirable, therefore, to obtain more data on this point. Consequently, the results from serum analyses of 40 weanling pups (20 fed diet 58-M, 80 ppm of magnesium; 20 littermates fed diet 58-M supplemented with an additional 100 ppm of magnesium) were compared. With the low intake of magnesium, the serum magnesium showed a highly significant depression ($P < 0.001$) from 1.8 ± 0.2 to 0.8 ± 0.2 mg per 100 ml (mean ± standard error), the serum inorganic phosphorus showed a significant elevation ($P < 0.05$) from 7.7 ± 0.4 to 8.7 ± 0.4 mg per 100 ml, and the serum calcium showed a highly significant depression ($P < 0.001$) from 11.4 ± 0.4 to 9.3 ± 0.4 mg per 100 ml.[5]

The following criteria of the development of the deficiency syndrome and lesions were used: weight gain; serum levels of magnesium; and aortic ash, calcium, and phosphorus content. The minimal magnesium requirement for the weanling dog was observed to be 140 ppm (dry-weight basis) in these studies.

SUMMARY

The minimal magnesium requirement of the weanling dog fed a semipurified diet was found to be approximately 140 ppm (dry weight basis).

Mineralized lesions were observed in the thoracic aorta of the animals fed the low

[5] Probability determined with Student's *t* test (Snedecor, '56).

magnesium. Upon analysis, the mineral was observed to consist largely of calcium and phosphorus. The amount of mineral accumulation in the aorta appeared to be a function of the magnesium content and duration of intake of the diet.

An elevation of blood serum inorganic phosphorus and a depression of serum calcium and magnesium appeared in the low-magnesium-fed (80 ppm) weanling dog, but no change in aortic magnesium content could be detected.

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The Mineral Requirements of the Dog[1]

IV. EFFECT OF CERTAIN DIETARY AND PHYSIOLOGIC FACTORS UPON THE MAGNESIUM DEFICIENCY SYNDROME

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The minimal magnesium requirement of the weanling dog has been shown to be 140 ppm (Bunce et al., '62) when the calcium, phosphorus, and fat levels were 0.6, 0.4, and 8.0%, respectively. Each of these nutrients and age have been reported to influence the magnesium requirement. Tufts and Greenberg ('38) first observed that elevation of dietary calcium and phosphorus aggravated the severity of the magnesium deficiency syndrome in the rat. Important investigations have been made more recently in various species by Colby ('51); Constant ('52); Hogan et al. ('50); House ('55); Hegsted et al. ('56); O'Dell ('57, '60); Selye ('58); Selye and Bajusz, ('58); Maynard et al. ('58) and McAleese and Forbes ('61). Evidence was presented by Vitale et al. ('59) that the magnesium requirement of the young rat was increased fourfold by feeding an atherogenic diet containing 20% of fat, 1% of cholesterol and 0.3% of cholic acid. Orent et al. ('32) noted that increased age retarded the development of magnesium deficiency symptoms in the young dog.

The studies to be reported here were designed to investigate the effect of these and other factors upon the magnesium deficiency syndrome of the weanling dog.

EXPERIMENTAL

A series of experiments were conducted in which dogs were fed a semipurified, low-magnesium basal ration no. 58-M, (80 ppm of magnesium) or the same ration following appropriate modifications in the concentration of one of the nutrients under study. A positive control was provided by a group fed the basal ration containing an additional 100 ppm of magnesium as anhydrous magnesium sulfate. Criteria used included rate of development and severity of the characteristic muscular and neural symptoms; weight gain; analyses of the blood serum for magnesium, calcium, and inorganic phosphorus, and of the aorta for total ash, calcium, and phosphorus. The effect of low-magnesium intake upon aortic ether extract and serum total cholesterol was investigated. The composition of the basal ration and the analytical procedures used have been described (Bunce et al., '62).

Approximately half of the pups were Beagles from our kennels, and the remainder were mixed Shepherd-Collie and Chow, purchased locally. Precautions were taken to protect the animals from distemper, infectious hepatitis, and *Leptospira canicola* infections and from internal and external parasites. The dogs were housed in expanded metal-bottom cages and fed the diets and distilled water ad libitum.

RESULTS AND DISCUSSION

Effect of variation in dietary calcium and phosphorus levels

Nine litters of weanling pups were distributed among 6 lots providing 6 or 7 pups per lot. Lot 1 was fed the low-magnesium basal ration which contained 80 ppm of magnesium, 0.6% of calcium, and 0.4% of phosphorus. Lot 2 was fed the same ration supplemented to contain 180 ppm of magnesium, an adequate amount for the wean-

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ling dog. Lots 3 and 4 were fed a low-magnesium basal ration in which the calcium had either been decreased to 0.3% (lot 3) or increased to 0.9% (lot 4) with the phosphorus level held constant at 0.4%. Lots 5 and 6 were fed a basal ration in which the phosphorus had either been decreased to 0.22% (lot 5) or increased to 0.9% (lot 6) with the calcium level held constant at 0.6%. The variations in the dietary content of magnesium, calcium, and phosphorus were achieved by adjusting the quantities of dietary anhydrous magnesium sulfate, calcium carbonate, disodium phosphate, and sucrose. The time of appearance of the acute stage of the magnesium deficiency syndrome varied between litters in a given dietary lot probably as a result of differences in such factors as average weight and age at weaning, and the expected size at maturity. For this reason, the duration of intake of the experimental diets was varied between 5 to 11 weeks, and an entire litter was sacrificed when the condition of the most severely affected pup in the litter was judged to be acute.

The characteristic visible syndrome of low-magnesium intake in the weanling dog, that is, anorexia, muscle weakness, and occasional ataxia and convulsions, occurred within three to six weeks in all pups fed a low-magnesium diet independent of the calcium or phosphorus content of the ration. The rate of appearance of these symptoms was generally accelerated in the animals in lot 6 (basal ration, 0.9% phosphorus), and retarded in lot 5 (basal ration, 0.22% phosphorus). The rate of gain in weight did not vary significantly with variations in dietary calcium and phosphorus although growth was retarded in all groups compared to lot 2 (180 ppm).

Postmortem inspection of the aortas revealed 100% incidence of lesions in animals that received low magnesium (80 ppm lot 1), low magnesium, high calcium (lot 4), and low magnesium and high phosphorus (lot 6). Lesions were observed in 4 of 6 pups in lot 3 (basal ration, low calcium), and 4 of 7 in lot 5 (basal ration, low phosphorus). In the latter, both the size and number of the lesions were greatly reduced, an observation supported by the results of chemical analyses. No lesions were detected in the lot 2 animals fed 180 ppm of magnesium.

The results of the aorta and serum analyses are presented in table 1. The average aortic calcium in the lot 2 animals was 95 mg per 100 gm of dry tissue. A sixfold increase to 560 mg per 100 gm of dry tissue was observed in their littermates fed the low-magnesium basal ration. Reduction of the dietary calcium had a slight effect since an average fivefold increase over the control level was noted in the lot 3 animals even though visible lesions had not been detected in two of the 6 pups of the lot. A tenfold increase in aortic calcium was observed in the pups of lot 4 fed low magnesium, high calcium.

Variation in the dietary phosphorus level produced more dramatic results. Although small lesions were visible in the aortas of 4 of 7 pups in lot 5 (basal ration, low phosphorus), the amount of calcium accumulation in the aorta was too small to raise the group average above the normal value of ash and Ca. Elevation of the dietary phosphorus of the basal ration to 0.9% resulted in a 13-fold increase over normal in the aortic calcium values of the animals in lot 6. The average aortic total ash and phosphorus content varied in the same direction as the calcium values but with lesser orders of magnitude. No changes were detected in the average aortic ether extract.

The average serum magnesium levels were depressed in all 5 groups fed a low-magnesium ration but no correlation was seen between the terminal serum magnesium and aortic calcium values with the exception of lot 5 (basal ration, low phosphorus). The majority of these animals had serum magnesium values above 1.0 mg per 100 ml and this group displayed both the highest average serum magnesium and the lowest average aortic calcium level of any lot fed a low-magnesium diet. A slight elevation of serum inorganic phosphorus and depression of serum calcium appeared in the animals fed the low magnesium. The average changes were greatest in those groups which showed the largest aortic mineral accumulations but this observation could not be extended to individual animals. Serum total cholesterol levels

TABLE 1

Effect of variation in dietary magnesium, calcium, and phosphorus on aortic, serum[1] mineral and on serum cholesterol content

Lot	1	2	3	4	5	6
No. of animals/lot	6	7	6	7	7	6
Dietary magnesium, ppm	80	180	80	80	80	80
Dietary calcium, %	0.6	0.6	0.3	0.9	0.3	0.6
Dietary phosphorus, %	0.4	0.4	0.4	0.4	0.22	0.9
No of animals showing aortic lesions	6	0	4	7	4	6
			Aorta analyses[2]			
Average ash, % dry-weight	3.07 ± 0.62	2.00 ± 0.09	2.80 ± 0.49	3.65 ± 0.62	2.01 ± 0.07	5.32 ± 1.17
Average calcium, mg/100 gm dry tissue	560 ± 255	95 ± 19	445 ± 202	950 ± 330	90 ± 30	1300 ± 280
Average phosphorus, mg/100 gm dry tissue	690 ± 230	290 ± 22	530 ± 106	820 ± 143	370 ± 43	1030 ± 261
Average ether extract, % dry-weight	10 ± 2	12 ± 3	7 ± 3	6 ± 1	8 ± 2	10 ± 2
			Serum analyses[2]			
Average magnesium, mg/100 ml	1.2 ± 0.2	1.8 ± 0.2	0.7 ± 0.2	1.2 ± 0.2	1.4 ± 0.3	0.9 ± 0.1
Average calcium, mg/100 ml	10.5 ± 1.4	11.7 ± 0.7	9.1 ± 0.8	8.8 ± 0.5	10.5 ± 0.9	8.5 ± 0.9
Average inorganic phosphorus, mg/100 ml	8.8 ± 0.6	7.3 ± 0.6	8.6 ± 0.7	9.8 ± 0.7	7.9 ± 0.4	9.8 ± 0.5
Average total cholesterol, mg/100 ml	330 ± 24	340 ± 33	300 ± 34	270 ± 23	300 ± 44	250 ± 33

[1] Terminal samples taken after 5 to 11 weeks on experiment.
[2] Mean ± standard error.

were not affected by changes in dietary magnesium, calcium, or phosphorus.

These data indicate that the level of dietary phosphorus exerted a definite influence upon the response of the weanling dog to a low-magnesium diet. It is reasonable to suggest, therefore, that the magnesium requirement of the pup varies with the level of dietary phosphorus as has been reported to occur in the guinea pig (O'Dell et al., '60) although alternative explanations are possible. An increase in the dietary calcium level from 0.6 to 0.9% caused an increase in the average amount of aortic ash, calcium and phosphorus, but did not affect other criteria. Reduction of the calcium content of the low-magnesium ration to 0.3% was of little benefit.

Effect of variation in dietary fat and cholesterol

Three litters of weanling pups were distributed into 5 groups of 4, 4, 3, 4, and 4 animals each for this investigation. Lot 1 animals were fed the basal ration containing 80 ppm of magnesium, 8% of fat, and no added cholesterol or cholic acid. The remaining groups were fed the basal ration with the following modifications: lot 2, 20% of fat; lot 3, 20% of fat + 1% of cholesterol + 0.3% of cholic acid; lot 4, 20% of fat + 180 ppm of magnesium; and lot 5, 180 ppm of magnesium. The fat was added as stabilized white grease[3] at the expense of sucrose. The animals were killed after 8 weeks of ad libitum consumption of the various rations, and the blood and aortas analyzed.

The pups in lots 4 and 5 were free from all deficiency symptoms and showed good weight gains throughout the entire experimental period. All had more than doubled their average initial weight of 3,000 gm by the 8th week. The animals fed the magnesium-low ration (lots 1, 2, and 3) showed an increase in weight comparable to the positive controls for the first three weeks. At that time, however, their weight gain

[3] A product of Oscar Mayer and Company, Madison, Wisconsin.

practically ceased and a constant weight of approximately 5,000 gm was maintained after the 5th week. Muscular weakness, occasional convulsions and ataxia were observed in these groups; however, no differences were detected between the groups fed low magnesium but different levels of fat or cholesterol.

Inspection of the thoracic aorta revealed the presence of the characteristic mineralized lesions in 10 of the 11 pups from lots 1, 2, and 3; the one exception occurred in group 3 fed the high fat, high cholesterol. No abnormalities were seen in the positive control lots. Staining of the aortas with Sudan IV failed to demonstrate the presence of atheromatous areas but an experimental period in excess of 8 weeks is generally required for the production of such lesions in the dog fed high fat, high cholesterol.

The average aortic calcium, phosphorus, and percentage of ash (table 2) was lower in the animals from lots 2 and 3 as compared with those in lot 1. The significance of this observation is doubtful in view of the small number of animals involved and the wide variation in individual values. A depression in blood serum magnesium and calcium and an elevation of blood serum inorganic phosphorus was seen in the low-magnesium fed pups but these changes were not altered by the dietary fat content. Total serum cholesterol values were elevated with the high-fat diets and rose even higher with the cholesterol-supplemented diet but these changes appeared to be independent of the level of dietary magnesium.

An elevation of dietary fat from 8 to 20% did not adversely affect either the rate of appearance or severity of the magnesium deficiency syndrome in the weanling dog, nor the changes in serum and aortic minerals associated with the deficiency. Supplying 180 ppm of magnesium was found to yield normal growth at both levels of dietary fat.

Effect of cholesterol and age on magnesium requirements in dogs

Young mature Beagle dogs ranging from 7 to 8 months of age were used in the first experiment. Two groups of three dogs were fed 80 ppm and 180 ppm of magnesium and two groups of 4 were fed replicates of the diets with the addition of 2% of cholesterol and 0.3% of cholic acid. These dogs were fed their respective diets from 12 to

TABLE 2

Effect of variation in dietary fat and cholesterol on aortic, serum, mineral and serum cholesterol[1] content

Lot	1	2	3	4	5
Dietary magnesium, ppm	80	80	80	180	180
Dietary fat, %	8	20	20	20	8
Cholesterol-cholic acid	—	—	+	—	—
No. of animals per lot	4	4	3	4	3
No. of animals showing aortic lesions	4	4	2	0	0
	Aorta analyses[2]				
Average ash, % dry-weight	3.28 ± 0.50	2.58 ± 0.40	2.29 ± 0.13	2.27 ± 0.10	2.17 ± 0.08
Average calcium, mg/100 gm dry tissue	526 ± 175	286 ± 135	178 ± 60	55 ± 10	56 ± 8
Average phosphorus, mg/100 gm dry tissue	529 ± 153	341 ± 60	292 ± 35	256 ± 19	269 ± 15
	Serum analyses[2]				
Average magnesium, mg/100 ml	0.9 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
Average calcium, mg/100 ml	7.9 ± 0.8	7.6 ± 0.7	7.4 ± 0.7	13.1 ± 0.3	12.0 ± 0.7
Average inorganic phosphorus mg/100 ml	9.3 ± 0.3	9.9 ± 0.2	9.9 ± 0.3	8.9 ± 0.1	9.1 ± 0.1
Average total cholesterol, mg/100 ml	280 ± 20	330 ± 25	446 ± 28	376 ± 32	326 ± 27

[1] Terminal samples taken after 8 weeks on experiment.
[2] Mean ± standard error.

TABLE 3

Effect of cholesterol and cholic acid on blood serum and aorta composition in mature dogs

Lot no.	Serum[1]			Aorta[1]			Bone[1]
	Mg	P	Cholesterol	Ca	Mg	P	Mg
	mg/100 ml serum			*mg/100 gm dry aorta*			*mg/100 gm bone ash*
1 80 ppm Mg	0.5±0.1	7.2±0.3	238±51	72±5	31±2	314±26	257±64
2 80 ppm Mg + cholesterol and cholic acid	0.7±0.1	5.5±1.7	476±37	54±10	38±11	276±14	363±38
3 180 ppm Mg	2.0±0.7	5.8±0.2	199±31	75±5	34±3	265±2	357±98
4 180 ppm Mg + cholesterol + cholic acid	1.5±0.4	5.8±0.4	363±21	74±2	35±3	297±19	434±78

[1] Mean ± standard error.

23 weeks. Weakness of the hind legs and convulsions were observed only in dogs fed the diet containing 80 ppm of magnesium with the addition of cholesterol and cholic acid after the 17th week. The analytical data are presented in table 3. No aortic abnormality or calcification was observed in any of the dogs. Aortic ash percentages in all 4 lots were essentially the same. Chemical analysis showed no change in aortic calcium.

In the case of serum magnesium, control lots fed 80 ppm of magnesium registered less than 0.8 mg per 100 ml magnesium, whereas those fed adequate levels averaged approximately 1.5 to 2 mg per 100 ml. Serum phosphate was increased in lot 1 animals with respect to the other lots. Cholesterol-cholic acid supplementation increased the serum cholesterol level by twofold in dogs fed a low-magnesium diet. This effect was reduced by an adequate level of magnesium.

The reduced serum cholesterol in lot 4 animals as compared to those in lot 2 and the appearance of magnesium deficiency symptoms only in lot 2 are suggestive of a relationship between cholesterol-cholic acid and magnesium metabolism in the mature dog.

A second experiment was made using two groups of dogs ranging in age from 1.5 years to 7 years of age. The rations were the same as used in other experiments, the low-magnesium one containing 80 ppm and the positive control, 180 ppm. These dogs were fed their experimental diets for 9 to 23 weeks. Body weight maintenance was depressed in those dogs fed the low-magnesium diet. Seven animals fed the low-magnesium basal ration had an average initial weight of 8,774 gm compared with 6,336 at the time the experiments were terminated. The initial weight of the 7 control dogs receiving 180 ppm of magnesium was 8,280 gm on the average as compared with their final average weight of 7,360 gm. There was no difference in the serum calcium and phosphorus of these dogs. The magnesium content of the serum averaged 0.95 mg per 100 ml for the low-magnesium group as compared with a serum magnesium content of 2.7 mg per 100 ml in those receiving 180 ppm. Likewise the average cholesterol was high in the low-magnesium-fed dogs (271 mg per 100 ml) in comparison with the average of the controls (203 mg per 100 ml). The aortic ash, calcium, and phosphorus was similar in both lots. Only one animal out of the 7 in the low-magnesium group showed any indication of aortic abnormalities. She was a 2.5-year-old dog in which a few calcified spots were observed on the aorta.

Studies with other nutrients

The effect upon the magnesium deficiency syndrome of several other substances was investigated in the same manner as before, using 4 to 6 animals per lot. The addition of 0.17% of either inorganic or elemental sulfur was without effect upon the previously listed criteria. A daily supplement of 50 mg of ascorbic acid or of 2.5 mg of menadione per animal was also without effect. Elevation of the potassium content of the low-magnesium diet from

0.6 to 1.2% by addition of potassium carbonate lowered the average amount of aortic calcium and phosphorus but there was wide individual variation and the benefit derived from this supplement was questionable. No effect was noted upon the general condition or weight gain of the pups or on the results of the serum determinations.

The addition of 250 ppm of fluorine as sodium fluoride to the low-magnesium basal ration caused a 50 to 75% reduction in weight gain as compared with the growth of littermates fed the basal ration alone during the first three weeks on experiment. Both groups failed to gain after that time whereas the growth of the positive control animals continued in a normal pattern. Although convulsions and muscular weakness occurred in the fluoride-fed animals and serum magnesium values were depressed to 1.2 mg per 100 ml, no aortic lesions were observed in these pups and the calcium, phosphorus and total ash content of the aortas were all within the normal range. It is possible that the severe restriction of growth and food intake in these animals was an important factor in preventing the accumulation of aortic mineral that is normally associated with magnesium deficiency in the weanling pup.

SUMMARY

The effects of certain dietary factors on the magnesium deficiency syndrome of the weanling dog have been studied. Both elevation of the dietary calcium from 0.6 to 0.9% and elevation of the dietary phosphorus from 0.4 to 0.9% were noted to increase the severity of the syndrome with the phosphorus effect being the more pronounced. Reduction of the dietary phosphorus to 0.22% was observed to alleviate the symptoms of magnesium deficiency but no differences were observed following a reduction in the dietary calcium to 0.3%.

Supplements of vitamin C, menadione, inorganic sulfate, or elemental sulfur were without effect on the magnesium deficiency syndrome.

Aortic ash, calcium, and phosphorus content were slightly diminished in the pup by increasing the dietary fat from 8 to 20% or by increasing the dietary potassium from 0.6 to 1.2%. No relief was observed with respect to the muscular and nervous symptoms or serum chemistry values. In mature dogs, the magnesium requirement appeared to fall between 80 and 180 ppm although a much longer depletion period was necessary than for the pup. The data suggested a relationship between cholesterol-cholic acid and magnesium metabolism in the mature dog.

Addition of 250 ppm of fluoride to the low-magnesium basal diet restricted food intake and growth, and prevented the appearance of aortic lesions and the accumulation of aortic mineral, but did not prevent the occurrence of muscular weakness and convulsions or the depression of serum magnesium.

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Liver Necrosis in Adult and Young Rats Fed a Protein-Free Diet Deficient in Vitamin E and the Effects of Certain Supplements and of Inanition[1,2]

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High-protein diets have a striking protective action against hepatoxic agents (Moise and Smith, '24; Goldschmidt et al., '39). Cystine and methionine are specific factors for preventing chloroform liver injury in protein-depleted dogs (Miller et al., '40). Vitamin E has been reported (Hove, '48) to prevent carbon tetrachloride poisoning in rats receiving low-protein diets.

When rats that were raised with a vitamin E-low ration are fed certain diets restricted in protein and vitamin E, dietary liver degeneration develops. Rats that do not die with necrotic liver develop chronic muscular dystrophy (Goettsch, '61). Alpha-tocopherol prevents both liver and muscle disorders. Among factors which prevent liver necrosis, but not muscular dystrophy, are selenium (Schwarz and Foltz, '57), cystine, methionine and increased dietary protein. The minimal amount of protein required for the induction of hepatic necrosis is unknown.

During a fast of 7 days in rats (Addis et al., '36) the liver loses 40% of its original protein, whereas the losses from carcass (muscle, skin and skeleton) are only 8%. The effect on liver cytoplasm (Kosterlitz, '47) and on muscle fibers (Roche and Hoerner, '33) of feeding rats a protein-free diet is similar to that of fasting. There is atrophy but no cellular degeneration.

This is a report on the rapid induction of dietary necrotic liver degeneration in adult and young vitamin E-deficient rats by protein-free diets, low in vitamin E, the prevention of this condition with certain supplements, and the effect of inanition.

EXPERIMENTAL

Diets. The 18.4% casein diet shown in table 1, when supplemented with vitamin E, supplied the minimal protein for normal growth, reproduction and lactation in the rat (Goettsch, '60). The protein-free diet contained minerals and vitamins but no added protein. One diet consisted only of sucrose. L-Cystine, DL-methionine and selenium (as sodium selenite) were incorporated in the diets and α-tocopheryl acetate, dissolved in corn oil, was given orally.

Procedure. The procedure of Goettsch ('61) was followed. Two groups of young rats were fed the necrogenic diets from the 21st day: those of females reared and bred with the commercial pellet ration; and those of females reared with the commercial pellet ration and given the vitamin E-low breeding diet (containing 26.6% of crude casein, 16.6% of yeast, 33.3% of cornstarch, 3.4% of salt mixture, 18.3% of lard and 1.8% of cod liver oil) during gestation and lactation. The latter young contained initial low stores of vitamin E. Adult vitamin E-deficient rats varying in age from 65 to 120 days and in body weight from 70 to 200 gm, were obtained by protecting young rats against necrotic liver degeneration during the early growth period with cystine or methionine supplements to the diets low in both vitamin E and protein or with the low vitamin E-adequate protein diet.

RESULTS AND DISCUSSION

Preparation of adult rats deficient in vitamin E. Young vitamin E-deficient rats were fed the low-protein diets, with and without supplements of L-cystine or of DL-

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methionine, at the concentrations shown in table 2. There was a high incidence of necrotic liver degeneration among rats fed the unsupplemented diets (Goettsch, '61). The sulfur-containing amino acids promoted growth, prevented liver necrosis in two-thirds of the animals and prolonged the period of survival before the onset of the liver disorder. The supplements were more effective at higher concentrations and cystine was more active than methionine (Weichselbaum, '35; Glynn et al., '45). The livers of rats receiving cystine appeared grossly to be more fatty. Ten rats fed the 18.4% casein diet grew normally (Goettsch, '60). Chronic muscular dystrophy appeared after 60 days in all of the rats that did not die with liver necrosis. Body weight and food intake decreased as the muscle disability progressed.

Of 112 rats (table 2) that received the sulfur-containing amino acids, 39 died with necrotic liver degeneration. Nineteen, one or more from each group, were under observation until death. Since the animals had difficulty in reaching food

TABLE 1

Composition of vitamin E-low diets

Constituents[1]	Adequate-protein 18.4% casein	Low-protein		Protein-free, no added protein
		8.3% casein	15% yeast	
	parts	*parts*	*parts*	*parts*
Crude casein[2]	18.4	8.3	—	—
Yeast[3]	—	—	15.0	—
Cornstarch	69.6	79.7	73.0	88.0
Salt mixture[4]	4.0	4.0	4.0	4.0
Lard[5]	6.0	6.0	6.0	6.0
Cod liver oil	2.0	2.0	2.0	2.0

[1] Synthetic vitamins added as supplement to each kilogram of adequate-protein (casein), low-protein (casein) and protein-free diets: (in milligrams) thiamine·HCl, 5; pyridoxine·HCl, 5; riboflavin, 10; niacin, 20; vitamin K, 5; PABA, 10; Ca pantothenate, 50; folic acid, 4; and biotin, 0.4. Half of the amount was added to the low-protein yeast diets.
[2] Casein, Grade B-1-F Crude, Casein Company of America, New York.
[3] Types of yeast used were Fleischmann, type 2019 and food yeast (*Torula utilis*, no. 3, locally grown).
[4] Salt mixture no. 2, USP, Nutritional Biochemicals Corporation, Cleveland.
[5] Locally obtained lard that had obviously poor keeping qualities.

TABLE 2

Protective action of cystine, methionine or increased dietary protein against necrotic liver degeneration in young vitamin E-deficient rats fed low vitamin E, low-protein diets

Vitamin E-low diet	Supplement[1]		Number of rats	Rats with necrotic liver		
	Substance	% of diet			Days of survival	
					Mean	Range
				%		
15% Fleischmann	0		16	100	10	5–25
15% Fleischmann	L-cystine	0.30	26	35	26	11–45
15% Fleischmann	L-cystine	0.60	6	17	19	
15% Fleischmann	DL-methionine	0.37	12	75	38	25–54
15% Fleischmann	DL-methionine	0.74	6	33	32	29–34
15% Torula	0		9	100	13	5–28
15% Torula	L-cystine	0.15	7	57	46	20–76
15% Torula	L-cystine	0.30	9	11	74	
15% Torula	L-cystine	0.60	6	0		
15% Torula	DL-methionine	0.37	12	25	28	16–33
15% Torula	DL-methionine	0.74	6	33	26	24–28
8.3% Casein	0		12	92	10	5–18
8.3% Casein	L-cystine	0.30	11	9	23	
8.3% Casein	DL-methionine	0.37	11	64	29	19–38
18.4% Casein	0		10	0		

[1] Concentrations of L-cystine and DL-methionine on basis of sulfur content.

there was frequently a terminal period of inanition. The rats died after 75 to 168 days of feeding, with normal liver and severe muscular dystrophy. Surviving rats and those fed the 18.4% casein diet were observed without change in diet for 65 to 120 days, at which time they presented moderate chronic muscular dystrophy. The body weight of these adult vitamin E-deficient rats varied from 70 to 200 gm.

Effect of feeding the protein-free diet to adult rats, deficient in vitamin E. The protein-free diet, low in vitamin E was fed to the adult vitamin E-deficient rats. Many of the rats died in two to 7 days (table 3). Twenty-five of 26 rats, given cystine during the early growth period, died after a mean period of 5 (2 to 13) days; of 12 rats that had been protected with methionine, all died after 28 (2 to 54 days); 8 of 10 rats that had been receiving the 18.4% casein diet died after 42 (12 to 61) days. At death the animals presented marked hemorrhagic necrosis of the liver with moderately fatty changes and severe muscular dystrophy.

The results with the protein-free diet differed from those observed in rats fed the low-protein diets (Goettsch, '51, '61). With low-protein diets: (1) the incidence of necrotic liver degeneration was only 15% in vitamin E-deficient rats with an initial body weight of 100 gm and the time of onset of the liver lesions was delayed to a mean of 70 days; (2) vitamin E-deficient rats that did not die with liver necrosis invariably developed severe muscular dystrophy and at death presented normal liver.

The rapid development of massive hepatic necrosis was observed by Glynn et al. ('45) in rats fed an amino-acid, sulfur-free diet after the rats had taken an amino acid, high methionine or a casein diet for about 4 months.

Supplementation of the protein-free diet with cystine or methionine. Supplementation of the protein-free diet with 0.30% of L-cystine or 0.37% of DL-methionine (concentrations on the basis of sulfur content) gave little or no protection to adult vitamin E-deficient rats (table 3). Cystine apparently protected 4 of 11 rats but methionine was without effect in 5 rats. Severe muscular dystrophy was noted in all of the rats at the time of death. These results indicate that under the given experimental conditions, hepatic necrosis in adult rats is not associated with the omission of the sulfur-containing amino acids from the diet.

Necrotic liver degeneration in young rats, deficient in vitamin E, with protein-free diets. All of the rats fed the protein-free diets shown in table 4 lost body weight. The incidence of necrotic liver degeneration and the time of survival before the onset of lesions were similar to those of rats receiving the low-protein diets (table 2) and were dependent upon the initial stores of vitamin E in the young rat (Goettsch, '61). Twelve rats, reared with pellets, died after a mean period of 28 days, whereas 41 of 43 young rats with low initial stores of vitamin E, died after a mean period of 10 days. The liver presented marked hemorrhagic necrosis with moder-

TABLE 3

Effect of feeding the vitamin E-low protein-free diet, with and without supplements, to vitamin E-deficient adult rats that apparently had been protected against liver necrosis during the early growth period

Protective agent during early growth period	Supplement to protein-free diet[1]		No. of rats	Rats with necrotic liver		
	Substance	% of diet			Days of survival	
					Mean	Range
				%		
Cystine	0		26	96	5	2–13
Methionine	0		12	100	28	2–54
Higher protein	0		10	80	42	12–61
Cystine	L-cystine	0.30	11	64	27	5–41
Methionine	DL-methionine	0.37	5	100	27	6–45

[1] Concentrations of L-cystine and DL-methionine on basis of sulfur content.

TABLE 4

Effect upon young vitamin E-deficient rats of vitamin E-low protein-free diets, with and without supplements, and of fasting

Diet	Supplement[1]		No. of rats	Rats with necrotic liver		
	Substance	% of diet			Days of survival	
					Mean	Range
				%		
Young reared with pellets						
Protein-free	0		12	100	28	21–35
Sucrose	0		12	0		
Young with initial low stores of vitamin E						
Protein-free	0		43	95	10	6–30
Sucrose	0		20	65	11	5–21
Protein-free	α-tocopheryl acetate[2]		12	0		
Protein-free	selenium[3]		12	0		
Protein-free	L-cystine	0.30	9	78	21	12–34
Protein-free	DL-methionine	0.37	9	100	28	11–47
Fasting	0		12	0		

[1] Concentrations of L-cystine and DL-methionine on basis of sulfur content.
[2] Two milligrams per rat per week.
[3] As sodium selenite, 0.5 ppm.

ate fatty changes. Although paresis had not been noted in the young rats the muscle was dystrophic. Two of the 43 rats died after 17 and 27 days with severe muscular dystrophy and normal liver.

Sucrose was fed to young rats (table 4). Twelve rats, reared with pellets, died after a mean period of 28 days without liver necrosis or muscular dystrophy. Of 20 young rats with initial low stores of vitamin E, 13 died with typical hepatic necrosis and 7 survived for a mean period of 25 days without liver or muscle lesions. The rats fed sucrose ate less than those receiving the protein-free diet.

Supplementation of the protein-free diet with α-tocopherol. Two milligrams of α-tocopheryl acetate per rat per week (table 4) gave complete protection against necrotic liver degeneration in 12 young vitamin E-deficient rats fed the protein-free diet. They survived for a longer time, 32 to 56 days, than the rats without vitamin E. There were mild fatty changes in the liver and slight atrophy in the muscle (Kosterlitz, '47; Roche and Hoerner, '33).

Supplementation with selenium. Supplementation of the protein-free diet with 0.5 ppm of selenium as sodium selenite (table 4) prevented necrotic liver degeneration in 12 young vitamin E-deficient rats. They survived for 22 to 32 days and presented normal liver and muscular dystrophy at death. The results were similar to those with low-protein diets (Goettsch, '61).

Supplementation with cystine or methionine. Supplementation of the protein-free diet with 0.30% of L-cystine or 0.37% of DL-methionine (concentrations on the basis of sulfur content) (table 4) gave little or no protection against necrotic liver degeneration to young vitamin E-deficient rats. Seven of 9 rats receiving cystine and all of the rats (9) given methionine died in 11 to 47 days. Hemorrhagic liver necrosis and severe muscular dystrophy were noted at death. The liver of rats fed cystine presented marked fatty infiltration. The results were similar to those observed in adult rats (table 3).

The failure of cystine or of methionine to prevent liver necrosis in rats fed the protein-free diet was not in agreement with the well-known protective action of the sulfur-containing amino acids with certain low-protein diets (table 2). The protective effect of cystine has been attributed by Schwarz and co-workers ('59) to contamination with traces of a Factor 3-active selenium compound. It is possible that the protective action of cystine or methionine supplements to the low-protein casein or yeast diets may be associated with the increase in available dietary protein. Rats fed the protein-free diet metabolize body protein, which theoretically is the ideal

protein for the rat. An ideal protein would not be supplemented by the sulfur-containing amino acids. Evidence is given by Lewis and Fajans ('51) that it was not possible to increase significantly the rate of growth of rats fed low-protein diets when either L-cystine or DL-methionine were added as a supplement to commercial lactalbumin.

Effect of fasting. Twelve young vitamin E-deficient rats (table 4) were subjected to fasting until death. The rats survived for a mean period of 9 (4 to 11) days. At death they presented atrophy of liver (Kosterlitz, '47) and of muscle.

Since liver necrosis occurred in rats fed the protein-free diet, or sucrose, but not in those subjected to fasting, it would appear that the calorie intake is an important factor in the induction of necrotic liver degeneration.

With certain low-protein diets, low in vitamin E, rats that do not die with necrotic liver develop chronic muscular dystrophy. They die, after a period of food restriction, with normal livers and muscular dystrophy (Goettsch, '61). Partial inanition may account for the occurrence of normal liver in these vitamin E-deficient rats with severe muscular dystrophy.

SUMMARY

Dietary protein is not essential for the induction of necrotic liver degeneration in the vitamin E-deficient rat. With a protein-free diet, low in vitamin E, hepatic necrosis and muscular dystrophy appear rapidly not only in young rats, but also in adults that have been protected during the early growth period with cystine or methionine supplements added to low-protein diets, or with increased dietary protein.

Addition of supplements to the protein-free diet had the following effects:

1. Alpha-tocopherol prevents the liver and muscle lesions.

2. Selenium prevents the liver, but not the muscle lesions.

3. L-cystine and DL-methionine have little or no protective action against liver or muscle lesions.

Liver necrosis and muscular dystrophy do not occur in young fasting rats that are deficient in vitamin E.

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Effects of Nonessential Amino Acids on the Growth of Vitamin B_6-Depleted Rats[1]

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The vitamin B_6 requirement of microorganisms is increased by the omission of nonessential amino acids from the growth medium (Snell, '56; Holden et al., '51; Lyman et al., '47) since, under these conditions, nonessential amino acids must be synthesized from their hydroxy- or keto-acid precursors by transamination or by other vitamin B_6-dependent reactions. Presumably, a dietary lack of nonessential amino acids could likewise increase the need of animals for vitamin B_6 because of the greater demand upon vitamin B_6-dependent reactions for the endogenous synthesis of amino acids. If this should be so, then a diet containing a mixture of nonessential amino acids might permit greater growth in vitamin B_6-depleted rats fed a limited amount of pyridoxine than would a diet that contained only a single nonessential amino acid.

The experiments reported here tested the effect of dietary nonessential amino acids upon the growth of vitamin B_6-depleted rats fed limited amounts of pyridoxine.

EXPERIMENTAL

Male rats of the Long-Evans strain, bred in our colony, were used in all experiments. One week before weaning of the young, the dams were fed a purified pyridoxine-deficient diet.[2] At weaning (three weeks of age) the young male rats were fed a vitamin B_6-deficient diet[3] until they were depleted of vitamin B_6 as judged by growth (Clarke and Lechycka, '43), i.e., failure to gain more than 3 gm in one week. Pyridoxine antagonists were not used. Under these conditions, two to three weeks were required to deplete weanling male rats of pyridoxine. Upon depletion, the rats were immediately transferred to the diets containing the amino acid mixtures. These diets were then fed ad libitum for two or three weeks. Vitamin supplements[4] were provided in castor cups three times weekly. Pyridoxine was supplied at a level of 3 μg per rat per day, an intake that is approximately 1/5th of the amount needed for maximal growth (Clarke and Lechycka, '43). The rats were individually caged in wire-bottom cages and were weighed three times weekly.

In addition to the amino acid mixtures, all the diets contained the following per 100 gm: cottonseed oil, 2.0 gm; salts (USP 14, '50), 4.0 gm; and powdered sucrose to 100 gm. Sucrose rather than dextrin or starch was used as the dietary carbohydrate in order to reduce vitamin synthesis by the intestinal flora. Differ-

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[2] Composition, per 100 gm diet: "vitamin-free" casein (Nutritional Biochemicals Corporation, Cleveland), 36.0; fat (Primex, Procter and Gamble, Cincinnati), 8.0; salts (USP 14, '50), 6.0; vitamins A, D, and E in cottonseed oil, 3.2; B-vitamins in sucrose, 2.0; powdered sucrose, 44.8. The following amounts of vitamins were supplied per 100 gm of diet: vitamin A, 3200 IU; vitamin D_2, 282 IU; and (in milligrams) DL-α-tocopheryl acetate, 16; thiamine·HCl, 0.86; riboflavin, 0.86; Ca D-pantothenate, 6.86; niacin, 3.43; *d*-biotin, 0.17; folic acid, 0.17; menadione, 0.99; and vitamin B_{12}, 4 μg.

[3] Composition, per 100 gm of diet: "vitamin-free" casein (Nutritional Biochemicals Corporation), 20.0; cottonseed oil, 5.0; salts (USP 14, '50), 4.0; choline bitartrate, 0.18; powdered sucrose, 70.82. Vitamin supplements were the same as in footnote 4, except that choline was omitted from the B-vitamin mix since it was incorporated into the diet.

[4] Two milliliters of a 20% ethanol solution of B-vitamins and menadione were fed three times weekly to provide the following intakes per rat per day: (in micrograms) thiamine·HCl, 43; riboflavin, 43; niacin, 171; *d*-biotin, 8.5; folic acid, 8.5; Ca D-pantothenate, 513; vitamin B_{12}, 0.21; menadione, 50; and choline chloride, 11 mg. Pyridoxine was added at the desired levels. Two drops of a solution of vitamins A, D, and E in cottonseed oil were fed three times weekly to provide 32 IU of vitamin A, 3.0 IU of vitamin D_2, and 162 μg of DL-α-tocopheryl acetate per rat per day.

ences in weight gain were evaluated by Student's *t* test.

RESULTS

An essential amino acid mixture based on whole-egg protein (Orr and Watt, '57) was used as the source of essential amino acids (table 1). Preliminary experiments with several mixtures of essential amino acids showed that the mixture patterned after egg protein was more satisfactory than the other mixtures tested.

This mixture of essential amino acids, which provided 0.95% N per 100 gm of diet, was supplemented with 0.55% of N from different combinations of amino acids (table 2). The mixture of 7 nonessential amino acids provided in the proportions of egg protein (Orr and Watt, '57) is shown as diet 101. In the other mixtures, each nonessential amino acid, with the exception of glutamic acid, was added initially at the level provided in diet 101. Glutamic acid was then added to bring the total nitrogen of each supplement to 0.55%. The results are presented in table 3.

TABLE 1

Essential amino acids per 100 gm of diet

		Millimoles available[1]	Mole ratios[2]
	gm		
L-Lysine·HCl	0.74	4.05	5.55
L-Histidine·HCl·H_2O	0.30	1.43	1.96
L-Arginine·HCl	0.73	3.46	4.74
L-Tryptophan	0.15	0.73	1.00
DL-Phenylalanine	0.53	3.21	4.40
L-Methionine	0.29	1.94	2.66
DL-Threonine	0.92	3.86	5.29
L-Leucine	0.81	6.17	8.45
L-Isoleucine (50%) with D-alloisoleucine (50%)	1.22	4.65	6.37
DL-Valine	1.37	5.85	8.01
$NaHCO_3$[3]	0.75		
Total N	0.95		
N in L-isomers[4]	0.75		
N in D-isomers[4]	0.20		

[1] Based on L- amino acids provided, except for DL-phenylalanine, whose D- form was considered equivalent nutritionally to the L- form.
[2] Mole ratios of L- amino acids to L-tryptophan as unity.
[3] The $NaHCO_3$ added was equivalent to the HCl provided by lysine, histidine and arginine.
[4] The N in D-phenylalanine is not included in this figure.

Experiment 1. In the first experiment, the supplement of 7 nonessential amino acids (diet 101) allowed significantly greater growth in two or three weeks than the following supplements: glutamic acid alone (diet 104); cystine and glutamic acid (diet 105); or a mixture of serine, cystine, and glutamic acid (diet 106).

Analysis of the weekly gains, however, showed that the greater cumulative gains produced by the supplement of the 7 nonessential amino acids of diet 101 resulted chiefly from the greater weight gain during the first week. During the second and third weeks, the supplement of glutamic acid or the supplement of cystine and glutamic acid permitted gains which were almost twice as great as the gains with these mixtures in the first week and which were comparable to the gain with the supplement of the 7 nonessential amino acids. On the other hand, the growth of the group fed the supplement of serine, cystine, and glutamic acid (diet 106) declined significantly during the second week in comparison with the gain for the first week. In the first week, the gain with this mixture was significantly greater than the gain with the

TABLE 2

Mixtures of nonessential amino acids (gm per 100 gm of diet)[1]

	Diet number											
	101	104	105	106	107	108	109	110	111	112	113	114
L-Cystine	0.22	—	0.22	0.22	—	—	—	—	—	0.22	0.22	—
DL-Serine	1.55	—	—	1.55	1.55	—	—	1.55	—	—	—	1.55
Glycine	0.33	—	—	—	—	0.33	1.11	0.33	2.22	1.11	2.22	0.33
L-Glutamic acid	1.14	5.78	5.48	3.31	3.57	5.10	3.57	2.93	1.43	3.31	1.13	1.40
L-Aspartic acid	0.65	—	—	—	—	—	—	—	—	—	—	0.65
L-Tyrosine	0.44	—	—	—	—	—	—	—	—	—	—	0.44
L-Proline	0.39	—	—	—	—	—	—	—	—	—	—	0.39

[1] All combinations provide 0.55 gm of N per 100 gm of diet.

TABLE 3

Effect of nonessential amino acids on the growth response of vitamin B_6-depleted rats to a daily intake of 3 μg of pyridoxine

Diet no.	Av. weekly weight gain[1]				Av. total weight gain	
	Average initial weight	1st week	2nd week	3rd week	2 Weeks	3 Weeks
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
			Experiment 1			
101 •	56 ± 2[1] (10)[2]	16 ± 1	11 ± 1	11 ± 1	28 ± 2	40 ± 2
104	65 ± 2 (10)	4 ± 1	10 ± 1	11 ± 1	14 ± 1	26 ± 2
105	64 ± 1 (10)	5 ± 2	9 ± 1	11 ± 1	14 ± 2	24 ± 2
106	60 ± 2 (10)	10 ± 1	6 ± 1	8 ± 1	16 ± 1	25 ± 1
			Experiment 2			
101	50 ± 2 (7)	13 ± 1	13 ± 1	—	25 ± 2	—
107	54 ± 2 (10)	7 ± 1	7 ± 1	—	14 ± 1	—
108	53 ± 1 (9)	6 ± 1	9 ± 1	—	16 ± 2	—
109	58 ± 2 (9)	10 ± 1	10 ± 4	—	20 ± 2	—
110	59 ± 2 (7)	7 ± 2	10 ± 1	—	17 ± 2	—
			Experiment 3			
101	53 ± 2 (9)	28 ± 2	9 ± 1	—	37 ± 2	—
109	56 ± 1 (13)	16 ± 1	9 ± 1	—	25 ± 1	—
111	55 ± 1 (12)	15 ± 1	8 ± 3	—	22 ± 1	—
112	54 ± 2 (10)	14 ± 2	9 ± 1	—	23 ± 2	—
113	57 ± 2 (8)	10 ± 2	7 ± 3	—	18 ± 2	—
			Experiment 4			
104	70 ± 2 (6)	−7 ± 2	11 ± 6(4)	—	5 ± 5(4)	—
107	73 ± 3 (7)	4 ± 2	9 ± 1	—	13 ± 2	—
109	69 ± 6 (7)	7 ± 3	10 ± 2	—	18 ± 3	—
114	76 ± 5 (6)	7 ± 2	13 ± 2	—	20 ± 2	—

[1] Mean and standard error of the mean.
[2] Figure in parentheses is the number of rats/group.

supplement of glutamic acid or with the supplement of cystine and glutamic acid.

The similarity between the weekly gains with the supplement of glutamic acid (diet 104) and the supplement of cystine and glutamic acid (diet 105) shows that a lack of cystine was not a primary limitation to growth under these conditions, despite the low level of methionine in the basal diet.

Experiment 2. In this experiment, the following supplements were tested: serine (1.55%) and glutamic acid (diet 107); glycine (0.33%) and glutamic acid (diet 108); glycine (1.11%) and glutamic acid (diet 109); serine, glycine (0.33%), and glutamic acid (diet 110). The higher level of glycine (1.11%) in diet 109 is equimolar with the level of DL-serine (1.55%) in diet 107. The supplement of the 7 nonessential amino acids (diet 101) was used as a control.

In a two-week period, the gain (20 ± 2 gm) produced by the supplement of glycine (1.11%) and glutamic acid (diet 109) was significantly greater than the gain (14 ± 1 gm) with the supplement of serine and glutamic acid (diet 107), but was not significantly greater than the gains with the supplement of glycine (0.33%) and glutamic acid (17 ± 2 gm). The gain produced by the supplement of the 7 nonessential amino acids (25 ± 2 gm) was again significantly greater than the gains with the other supplements.

In the first week, the supplements of serine and glutamic acid, glycine (0.33%) and glutamic acid, and serine, glycine (0.33%), and glutamic acid produced similar gains (6 to 7 gm). The gain with the supplement of glycine (1.11%) and glutamic acid was significantly greater (10 ± 1 gm) and did not differ significantly from the gain (13 ± 1 gm) with the supplement of 7 amino acids.

In the second week, the gain (10 ± 4 gm) with the supplement of glycine (1.11%) and glutamic acid was still superior to that (7 ± 1 gm) observed with the supplement

of serine and glutamic acid, but was not significantly greater than the gains with the other two supplements (9 to 10 gm). The gain (13 ± 1 gm) with the supplement of the 7 amino acids was significantly greater than the gains with the other combinations, with the exception of the mixture of glycine (1.11%) and glutamic acid (diet 109).

Experiment 3. The observation of the previous experiment that the weight gain with glycine (1.11%) and glutamic acid was approximately 75% of that with the supplement of 7 nonessential amino acids suggested a need for glycine under these conditions. Hence, it was possible that a higher level of glycine might be even more effective. In this experiment, comparisons were made of supplements containing 1.11 and 2.22% of glycine, with and without the addition of 0.22% of cystine.

Increasing the level of glycine from 1.11 to 2.22% did not improve growth (diets 109 and 111). The addition of 0.22% of cystine to 1.11% of glycine was also ineffective (diet 112). The addition of 0.22% of cystine to 2.22% of glycine significantly depressed growth (diet 113). The weight gains with each of the supplements for the first week were greater than the gains for the second week, in which the gains with all of the supplements were quite similar. The differences in gains for the two-week period reflect the differences in the gains for the first week.

In the first week, the gains with the supplement of the 7 amino acids and with the supplement of glycine (1.11%) and glutamic acid were significantly greater than the gains with these supplements in the first week of the preceding experiment although the time required for vitamin B_6 depletion and the weights of the rats at depletion were similar in both experiments. The difference, which is assumed to represent the relative need of these animals for these supplements in the first week, could not be related to the growth of the rats during the vitamin B_6 depletion period.

Experiment 4. A 4th experiment compared the following amino acid supplements: glycine (1.11%) and glutamic acid (diet 109); glycine, serine, proline, tyrosine, aspartic acid, and glutamic acid (diet 114); and serine (1.55%) and glutamic acid (diet 107). The supplement of glutamic acid alone (diet 104) was used as a control. The supplement of the 6 amino acids was based on the supplement of 7 amino acids previously used (diet 101), but cystine was omitted, and additional glutamic acid was added to bring the level of nitrogen up to 0.55%.

With the supplement of glycine and glutamic acid (diet 109), the gain for two weeks (18 ± 3 gm) equaled the gain (20 ± 2 gm) produced by the supplement of 6 amino acids (diet 114). With the supplement of serine and glutamic acid (diet 107), the gain for two weeks (13 ± 2 gm) was significantly less than the gain with the supplement of 6 amino acids. The difference in the gains with the supplement of serine and glutamic acid (diet 107) and the supplement of glycine and glutamic acid (diet 109) was not significant although the latter supplement allowed a greater gain in the first week.

The most striking observation in the first week was the very poor growth of the rats fed the supplement of glutamic acid alone (diet 104). None of the rats gained weight, and two rats died after the first week. In the second week, the weight gains of the 4 survivors were zero, 8, 8, and 28 gm.

The group fed the supplement of serine and glutamic acid (diet 107) gained significantly more weight in the second week than in the first week. The other two groups (diets 109 and 114) also gained more weight in the second week than in the first week, but the difference was not significant.

These results indicate that provision of a mixture of nonessential amino acids including glycine and serine significantly increased the growth of vitamin B_6-depleted rats in the first week of pyridoxine repletion. Also, if comparison is made with the preceding experiments, it appears that the omission of cystine from the "complete" mixture of 7 nonessential amino acids significantly decreased the weight gain in the first week although the addition of the same amount of cystine to supplements of glutamic acid, glycine and glutamic acid, or serine and glutamic acid did not improve growth.

DISCUSSION

The most interesting observation of these experiments is the difference in the weight gains produced by the different supplements during the first week and the lessening of this difference in the second and third weeks. For example, in the first experiment, comparison of the supplement of 7 nonessential amino acids with the supplement of glutamic acid shows that the greater weight gain in two weeks with the former supplement resulted chiefly from the larger gain in the first week (16 ± 1 gm vs. 4 ± 1 gm). In the second and third weeks, comparable gains were observed with both supplements.

The larger gain produced by the supplement of 7 nonessential amino acids in the first week could be explained by any one or any combination of the following. (1) The presence of nonessential amino acids in the diet may have decreased the need for their endogenous synthesis by reactions requiring pyridoxal phosphate. Thus, since less of the limited amount of dietary pyridoxine was needed for these reactions, more was available for other functions requiring pyridoxal phosphate, i.e., supplying nonessential amino acids in the diet "spared" pyridoxine. (2) The change from the 20% casein diet fed in the depletion period to the amino acid diet containing several nonessential amino acids may have required less enzymatic adaptation to the synthesis of nonessential amino acids than did the change to the diets containing fewer nonessential amino acids. (3) The addition of nonessential amino acids may have relieved an amino acid imbalance created by the omission of nonessential amino acids except glutamic acid (Kumta and Harper, '60). (4) Provision of nonessential amino acids in the diet may have altered the microbial population of the intestine to provide a more beneficial environment for the vitamin B_6-depleted rat through increased synthesis of vitamin B_6 or other unknown factors. The role of coprophagy cannot be evaluated. No special efforts were made to prevent coprophagy, and postmortem examination invariably revealed fecal material in the stomach.

All of these effects are possible, and the data do not differentiate among them. On the other hand, the quite comparable weight gains observed with all of the supplements in the second or third weeks show that the differences in the nonessential amino acid composition of the supplements had relatively little effect on growth after the first week.

It would be expected, from bacterial studies (Holden et al., '51), that any diet that decreased the need for the synthesis of nonessential amino acids in an animal fed a limited amount of pyridoxine would "spare" pyridoxine for other functions, especially growth. The greater gains, however, in the second and third weeks with the supplements containing only a few nonessential amino acids and the smaller gains with the supplement of 7 nonessential amino acids suggests that the following conditions may have arisen after the first week. (1) Although the supplement of 7 nonessential amino acids was initially beneficial for the reasons previously mentioned, an imbalance of essential amino acids in the basal diet may have nullified any possible benefit from the supplement after the first week. Since the pyridoxine provided by the vitamin supplement remained constant although food intake increased as the rats grew larger, an increased intake of an unbalanced amino acid mixture would increase the need for vitamin B_6 in the catabolism of the amino acids supplied in excess. Similar studies with other mixtures of essential amino acids should help clarify the role of essential amino acids in the effects observed here. (2) The amount of vitamin B_6 produced by microbial synthesis and available to the rat may have increased to a greater extent in the rats fed the supplement containing only a few nonessential amino acids. The pyridoxine intake of 3 μg per day was a primary limitation to growth in these experiments. Other experiments in this laboratory have shown that the weight gains of rats fed the basal diet with the supplement of 7 nonessential amino acids (diet 101) increase as the pyridoxine intake increases, up to a daily intake of 10 μg. A daily intake of 10 μg of pyridoxine will also produce nearly maximum weight gains in vitamin B_6-depleted rats fed a 20% casein diet (Clarke and Lechycka, '43).

The greater gains with the supplements containing glycine or serine in combination with glutamic acid, in comparison with the supplement of glutamic acid alone, may be another indication of the importance of glycine in the metabolism of the vitamin B_6-deficient rat (Gershoff and Faragalla, '59). The somewhat smaller gain with DL-serine than with an equimolar amount of glycine could be the result of the less efficient use of the D-isomer of serine.

The apparent ineffectiveness of cystine in some of the supplements tested, despite the low methionine content of the basal diet, suggests that other inadequacies of the diet were more limiting than the need for cystine. This possibility is supported by a comparison of the supplement of 7 nonessential amino acids, including cystine (diet 101), with the supplement of 6 nonessential amino acids which lack cystine (diet 114). Although the levels of glutamic acid in the two supplements differ slightly, the greater gain with the former mixture in the first week may be attributed to the addition of cystine.

SUMMARY

Experiments were carried out to study the effect of the nonessential amino acid composition of the diet on the growth of rats fed a limited amount of pyridoxine. It was postulated that the growth of rats fed a diet supplemented with a single nonessential amino acid, together with a limited amount of pyridoxine, would be less than that observed in rats fed an isonitrogenous diet containing a mixture of nonessential amino acids, since presumably, the need for pyridoxine with the former diet would be increased by the greater demands placed upon reactions requiring pyridoxal phosphate for the endogenous synthesis of nonessential amino acids.

Male rats, previously depleted of vitamin B_6, were fed ad libitum diets of purified L- and DL-amino acids which supplied 0.95% of nitrogen from essential amino acids and 0.55% of nitrogen from nonessential amino acids. The essential amino acids were supplied in the ratios present in egg protein. The intake of pyridoxine was 3 μg per rat per day.

During the first week of pyridoxine supplementation, a mixture of 7 nonessential amino acids, in the ratios present in egg protein (glutamic acid, aspartic acid, glycine, serine, cystine, proline and tyrosine), permitted the greatest weight gains. Glutamic acid alone allowed only poor growth. Serine and glutamic acid caused some improvement over glutamic acid alone, but a combination of glycine and glutamic acid significantly improved growth. The combination of glycine and glutamic acid was as effective as a combination of glutamic acid, aspartic acid, glycine, serine, proline, and tyrosine. After the first week of pyridoxine supplementation, however, growth was affected much less by variations in the nonessential amino acid composition of the diet.

The results suggest that nonessential amino acids may "spare" vitamin B_6 under certain conditions.

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Lactose Diets and Cholesterol Metabolism

II. EFFECT OF DIETARY CHOLESTEROL, SUCCINYLSULFATHIAZOLE AND MODE OF FEEDING ON ATHEROGENESIS IN THE RABBIT[1]

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An elevation in serum and hepatic cholesterol concentration and potentiation in severity of atherosclerosis was observed in rabbits that received a 0.35% cholesterol-containing diet in which lactose (29.35%) was substituted for an equal amount of sucrose (Wells and Anderson, '59). It then seemed worthwhile to determine the effect on sterol metabolism of feeding rabbits varying amounts of cholesterol in sucrose- or lactose-containing diets. Studies conducted with the rat as the experimental animal supported the hypothesis that lactose acts by influencing bile acid metabolism. Thus, the size of the enterohepatic bile acid pool in the lactose-fed rat was observed to be larger than that of the sucrose-fed controls (Wells et al., '60; Portman, '60). The effect of feeding lactose to experimental animals tended to parallel that obtained when antibiotics were added to the diet of rats (Lindstedt and Norman, '56) or when these animals were reared in an environment free of germs (Gustafsson et al., '57). Accordingly, the influence of lactose and succinylsulfathiazole[2] (SST) on the serum and liver cholesterol concentration and degree of atherosclerosis was compared in the rabbit.

During the course of our studies on the effect of adding various levels of dietary cholesterol to lactose- or sucrose-containing diets, we investigated the effect of feeding rabbits basal diets containing either a moderate level of cholesterol or no cholesterol for alternate periods of 24 hours. Another experiment was also conducted in which the number and length of the feeding periods were restricted. These latter experiments were similar to those conducted by Cohn and Joseph ('60) in the rat except that our interest was in the response of cholesterol-fed rabbits to the restricted-eating schedule. Cohn and co-workers ('60) extended their experiments in the rat to the cholesterol-fed chicken and reported that the serum cholesterol level and atherogenesis of the restricted-fed chickens was significantly higher than that of the ad libitum-fed controls. Our present observations in the rabbit are in agreement with the previous observations in the chicken.

METHODS AND EXPERIMENTAL

Dietary cholesterol variation. Male rabbits of the New Zealand White strain weighing 1,000 to 1,500 gm were divided into 12 groups of 5 to 8 animals and fed the lactose- or sucrose-containing diets reported previously (Wells and Anderson, '59). Although all groups in this series were not fed simultaneously, the lactose-fed groups were always tested experimentally with a comparable sucrose-fed group. For one series (two groups), the animals were fed only trace amounts of cholesterol (estimated as 0.05% by weight) derived from the dietary cod liver oil (2%) for a period of 13 weeks. In other experiments of 8 weeks' duration, the rabbits received the following amounts of cholesterol in their diets at the expense of cellulose:[3] one series, 0.2%, two series, 0.35%

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[2] Sulfasuxidine ®. Merck and Company, Rahway, New Jersey.
[3] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

and two series, 0.5%. The rabbits were housed individually and fed water and diet, ad libitum. The diet intake and weekly body weight for each animal were recorded. Animals were electrocuted, the blood removed, and the serum and liver cholesterol concentrations and degree of thoracic arch atherosclerosis evaluated by methods previously reported (Wells and Anderson, '59). The animals that received trace amounts of cholesterol in their diet were examined for serum cholesterol concentration only.

SST feeding. Two identical series were conducted to compare the effect of dietary lactose and SST on cholesterol-induced atherosclerosis in the rabbit. In the first series, 0.5% of SST was added at the expense of cellulose, whereas in series 2, 1.0% of SST was used. Each series consisted of 4 groups of 4 to 8 male rabbits of the New Zealand White strain weighing 1,000 to 1,200 gm. Groups A and B were fed the sucrose- or lactose-0.35% cholesterol diets, respectively, for a period of 8 weeks (Wells and Anderson, '59). Groups C and D were fed diets identical to those consumed by groups A and B, respectively, with the exception of the added SST. The animals were treated as described in the previous section and serum and hepatic cholesterol concentrations were determined in the usual manner.

Mode of feeding. Male rabbits of the New Zealand White strain weighing 1,000 to 1,200 gm were divided into 9 groups of 5 to 7 animals. The animals in series 1 (5 groups) received the sucrose-containing diet (0.35% cholesterol) previously described and the groups in series 2 received the basic lactose-containing diet (0.35% cholesterol) for 8 weeks. In both series, group A was the ad libitum-fed control, group B and/or B-1 (series 1 only) were restricted to two or three one-hour feeding periods, respectively. Diet was provided the group B animals between 9:00 to 10:00 AM and 5 to 6 PM and to those in group B-1 for an additional period at 12:00 to 1:00 PM. No attempt was made to pair-feed the experimental animals but food intake was recorded in all experiments. In both series, the animals in groups C and D were given appropriate diets containing no cholesterol or 0.7% of cholesterol on a daily alternation schedule.

RESULTS

Dietary cholesterol variation. After 13 weeks of ingesting diets essentially free of cholesterol, the total serum cholesterol values of the lactose-fed rabbits were somewhat higher than those observed in the sucrose-fed group (table 1, group 2, 113 ± 88 mg per 100 ml vs. group 1, 72 ± 36 mg per 100 ml). When the level of dietary cholesterol was increased to 0.2%, the difference in total serum and liver cholesterol content between sucrose- and lactose-fed rabbits was found to be more significant (table 1, group 3, serum, 493 ± 68 mg per 100 ml and liver 24.4 ± 6.6 mg per gm[4] vs. group 4, serum, 722 ± 477 mg per 100 ml and liver, 36.3 ± 20.4 mg per gm). The variation in the response of the two dietary groups reached a maximum when 0.35% of cholesterol was added to the diets (table 1, e.g., group 5, serum, 685 ± 28 mg per 100 ml and liver, 26.6 ± 12.2 mg per gm vs. group 6, serum, 1921 ± 421 mg per 100 ml and liver, 110.6 ± 28 mg per gm). There was no distinction, however, in sterol values between those rabbits fed sucrose- or lactose-supplemented diets when the added cholesterol was increased to 0.5% of the diets (table 1, e.g., group 9, serum, 1,850 ± 514 mg per 100 ml and liver, 71.8 mg per gm vs. group 10, serum, 1,846 ± 365 mg per 100 ml and liver, 71.7 ± 22.7 mg per gm). The atherosclerosis score was higher for the lactose-fed groups than for their corresponding sucrose-fed controls only when the cholesterol content of the diet was low or moderate (0.35%). The difference in severity of atherogenesis due to the carbohydrate variation was not observed when 0.5% of cholesterol was added to the diet (table 1, groups 9–12).

SST feeding. The sterol economy of rabbits fed 0.5 or 1.0% of SST was noted to be similar to that of lactose-fed animals (table 2, e.g., series 1, total serum cholesterol, group A (sucrose), 585 ± 148 mg per 100 ml, group B (lactose) 1,249 ± 446 mg per ml and group C (0.5% SST), 1,305 ± 104 mg per 100 ml). The effect of both lactose- and SST-feeding, (group D), resulted in slightly higher values than for either lactose or SST alone, 1,555 ± 292 mg per 100 ml; however, the two supple-

[4] Mg/gm indicates milligrams/gram of dry, fat-free tissue.

TABLE 1

Effect of dietary cholesterol variation on serum and liver cholesterol concentration and atherosclerosis in lactose- and sucrose-fed rabbits

Group[1] no.	No. animals	Diet[2]	Dietary cholesterol	Average daily food intake	Serum cholesterol		Liver cholesterol		Atherosclerosis score[3]
					Free	Total	Free	Total	
			%	*gm*	*mg/100 ml*	*mg/100 ml*	*mg/gm*[4]	*mg/gm*[4]	% (range)
1	5	Sucrose	0	50.9	15 ± 15[5]	72 ± 36[5]	—	—	—
2	7	Lactose	0	52.3	39 ± 43	113 ± 88	—	—	—
3	8	Sucrose	0.2	60.3	134 ± 67	493 ± 68	10.1 ± 2.1[5]	24.4 ± 6.6[5]	5(0–65)
4	8	Lactose	0.2	60.8	210 ± 43	722 ± 477	12.6 ± 2.2	36.3 ± 20.4	28(0–65)
5	6	Sucrose	0.35	54.6	195 ± 111	685 ± 422	10.9 ± 1.6	26.6 ± 12.2	9(0–40)
6	6	Lactose	0.35	52.8	662 ± 233	1921 ± 421	23.0 ± 4.6	110.6 ± 28	48(5–80)
7	8	Sucrose	0.35	55.2	226 ± 75	788 ± 488	9.6 ± 2.7	35.4 ± 11.2	12(0–50)
8	7	Lactose	0.35	53.4	379 ± 235	1161 ± 625	13.8 ± 3.2	67.7 ± 17.7	35(5–85)
9	7	Sucrose	0.5	55.1	642 ± 158	1850 ± 514	14.9 ± 4.2	71.8 ± 24.2	46(5–80)
10	7	Lactose	0.5	51.5	656 ± 165	1846 ± 365	16.6 ± 2.6	71.7 ± 22.7	45(0–100)
11	7	Sucrose	0.5	55.1	550 ± 85	1743 ± 306	14.9 ± 0.9	76.8 ± 27.6	78(60–95)
12	6	Lactose	0.5	48.7	651 ± 133	1842 ± 324	20.5 ± 4.4	133.5 ± 30.2	78(50–95)

[1] Male rabbits of the New Zealand White strain; experimental period for groups 1 and 2 was 13 weeks, for groups 3–12, 8 weeks.
[2] See text for description of diet.
[3] Thoracic arch score, zero to 100% involvement.
[4] Milligrams per gram of dry, fat-free tissue.
[5] Standard deviation, $\sigma = \sqrt{\Sigma X^2 - \frac{(\Sigma X)^2}{N}/N - 1}$.

TABLE 2

Comparison of effect of feeding lactose, sucrose and SST[1] on serum and liver cholesterol concentration and atherosclerosis in the rabbit

Group no.	No. animals	Diet[2]	Average daily food intake	Serum cholesterol		Liver cholesterol		Atherosclerosis score[3]
				Free	Total	Free	Total	
			gm	*mg/100 ml*	*mg/100 ml*	*mg/gm*[4]	*mg/gm*[4]	*% (range)*
Series 1								
A	4	Sucrose	64.3	140 ± 10[5]	585 ± 148[5]	13.8 ± 1.7[5]	43.1 ± 8.5[5]	15(5–20)
B	5	Lactose	58.5	394 ± 77	1249 ± 446	16.3 ± 3.6	81.7 ± 6.3	44(20–60)
C	7	Sucrose + 0.5% SST	63.3	371 ± 127	1305 ± 104	14.8 ± 3.5	65.3 ± 21.0	58(25–90)
D	6	Lactose + 0.5% SST	59.8	490 ± 65	1555 ± 292	13.8 ± 3.1	73.9 ± 31.0	81(70–95)
Series 2								
A	8	Sucrose	55.2	226 ± 75	788 ± 477	9.6 ± 2.7	35.4 ± 11.2	12(0–50)
B	7	Lactose	53.4	379 ± 235	1161 ± 625	13.8 ± 3.2	67.7 ± 17.7	35(5–80)
C	5	Sucrose + 1% SST	55.3	399 ± 190	1273 ± 459	13.5 ± 3.5	60.1 ± 27.0	45(15–70)
D	4	Lactose + 1% SST	45.9	375 ± 118	1318 ± 448	12.5 ± 3.1	64.2 ± 26.0	69(55–90)

[1] Succinylsulfathiazole.
[2] All diets contain 0.35% of cholesterol, composition previously reported (Wells and Anderson, '59). Sulfasuxidine was added at the expense of cellulose. Experimental period was 8 weeks.
[3] Thoracic arch score, zero to 100% involvement.
[4] Milligrams per gram of dry, fat-free tissue.
[5] Standard deviation, see footnote 5, table 1.

ments did not produce an additive effect. Parallel results were observed in the case of total liver cholesterol concentrations; group A, 43.1 ± 8.5 mg per gm, group B, 81.7 ± 6.3 mg per gm, group C, 65.3 ± 21 mg per gm and group D, 73.9 ± 31 mg per gm. The atherosclerosis score was always found to be somewhat higher in the animals fed SST than in those receiving lactose.

Mode of feeding. Rabbits receiving the sucrose-cholesterol-containing diet for two or three one-hour periods daily for 8 weeks exhibited significantly higher serum and hepatic cholesterol concentration and atherogenesis than rabbits that were allowed free access to the diet (table 3), total serum cholesterol, group A (ad libitum) 685 ± 422 mg per 100 ml vs. group B, (two feeding periods), 1,390 ± 222 and group B-1 (three feeding periods) 1,434 ± 291 mg per 100 ml and total liver cholesterol concentration, group A, 26.6 ± 12.2 mg per gm vs. group B, 44.9 ± 4.8 mg per gm and group B-1, 45.8 ± 4.3 mg per gm. The increased sterol levels resulting from this feeding procedure were not observed, however, when the diet contained lactose (table 3, series 2). The difference in the sterol metabolism of the groups fed 0.0 and 0.7% cholesterol-containing diets alternately was not unlike that observed commonly for sucrose vs. lactose-fed rabbits supplemented with a 0.35% dietary cholesterol (table 3, groups C and D vs. group A in both series). An exception was the serum cholesterol value for sucrose-fed rabbits (series 1, group C, 1,495 ± 687 mg per 100 ml and group D, 1,103 ± 670 mg per 100 ml vs. group A, 685 ± 422 mg per 100 ml). The consumption of the 0.0 and the 0.7% cholesterol-containing diets by the animals in groups C and D was approximately equivalent.

DISCUSSION

The greatest variation in cholesterol metabolism in the rabbits fed sucrose- or lactose-containing diets occurred at a crucial level of cholesterol in the diet, namely 0.35%. Whether the absence of a "lactose effect" at the 0.5 % cholesterol level is due to the saturation of the absorption mechanism or the exhaustion of available bile salts in the lumen of the intestine or an

TABLE 3

Effect of restricted eating and dietary cholesterol on serum and liver cholesterol concentration and atherosclerosis in the rabbit

Group no.	No. animals	Diet schedule[1]	Average daily food intake	Serum cholesterol		Liver cholesterol		Atherosclerosis score[2]
				Free	Total	Free	Total	
			gm	*mg/100 ml*	*mg/100 ml*	*mg/gm*[3]	*mg/gm*[3]	*% (range)*
Series 1 Sucrose								
A	6	Ad libitum	54.6	195 ± 111[4]	685 ± 422	10.9 ± 1.6[4]	26.6 ± 12.2[4]	9(0–40)
B	5	Restricted, 2 periods	46.7	436 ± 90	1390 ± 222	11.6 ± 1.8	44.9 ± 4.8	33(5–70)
B-1	7	Restricted, 3 periods	55.2	445 ± 109	1434 ± 291	12.4 ± 2.4	45.8 ± 4.3	12(0–80)
C	7	Alternate daily	68.1	426 ± 180	1495 ± 687	12.4 ± 1.9	48.9 ± 16.2	19(3–70)
D	7	Alternate daily	60.5	331 ± 225	1103 ± 670	12.1 ± 2.0	34.7 ± 6.2	21(0–90)
Series 2 Lactose								
A	6	Ad libitum	52.8	662 ± 233	1921 ± 421	23.0 ± 4.6	110.6 ± 28.0	48(5–80)
B	6	Restricted, 2 periods	46.0	519 ± 291	1701 ± 597	29.8 ± 4.4	124.6 ± 59.0	46(5–80)
C	6	Alternate daily	60.4	533 ± 132	1892 ± 441	16.1 ± 2.6	96.6 ± 37.0	52(10–80)
D	7	Alternate daily	50.2	624 ± 136	1798 ± 330	32.8 ± 14.9	157.0 ± 41.0	47(15–80)

[1] The diets for groups A, B, and B-1 in both series consisted of the basic 0.35% cholesterol containing diet (see text for description); groups C and D received diets containing 0.0% and 0.7% of cholesterol on alternate days. All experimental periods were 8 weeks.

[2] Thoracic arch score, zero to 100% involvement.

[3] Milligrams per gram of dry, fat-free tissue.

[4] Standard deviation, see footnote 5, table 1.

alternate explanation is difficult to ascertain. Increased intestinal motility has been postulated as one of the mechanisms that could explain the hypercholesterolemia associated with lactose-feeding (Wells and Anderson, '59; Portman, '60). A possible result of this mechanism might be an increased rate of enterohepatic circulation of bile acid. This effect combined with the decrease in the breakdown of bile acids by the intestinal flora could result in a conservation of bile acids and thereby secondarily result in an increased absorption of dietary cholesterol. The influence of diet on intestinal microorganisms and the ability of certain microorganisms to modify bile acid structure has been extensively studied by Lindstedt and Norman ('56), Gustafsson et al. ('57), Portman, et al. ('55) and Portman ('60). If the "lactose effect" can be explained primarily on the basis of fecal excretion of bile acids, this effect ought to be lessened by conducting the experiment under germ-free conditions. Similarly, any lactose mechanism not related to intestinal microorganisms should function even in the presence of "intestinal antibiotics." The failure to observe an additive effect of lactose and SST on the cholesterol levels of rabbit serum and liver tissue tends to support a "lactose effect" mechanism associated with intestinal microorganisms. Pertinent results were obtained by Kritchevsky et al. ('59) for chickens reared under germ-free conditions. Thus, the hypocholesterolemic effect of feeding glucose or starch in a conventional environment was partially destroyed. An analogous effect was noted when an antibiotic was added to glucose-cholesterol diets (Kritchevsky et al., '58). Elevated serum cholesterol levels have been reported to occur as a result of feeding neomycin sulfate to rats[5] and to rabbits (Fisher, '60). The opposite effect resulted from the addition of neomycin to the diet of human subjects (Samuel and Steiner, '59).

In the present study, rabbits were given the opportunity to develop reduced cholesterol concentrations by consuming a cholesterol-free diet every other day. The results suggest that the rabbit is unable to benefit from the omission of dietary cholesterol for only 24 hours. This observation may be explained in part by no attempt having been made to prevent coprophagy.

Cohn ('60) has suggested a significant role for the daily pattern of ingestion of a diet in the regulation of body metabolism. Further communications (Cohn et al., '60)[6] have revealed that atherosclerosis in the chicken was more pronounced when the atherogenic diet was eaten under restricted conditions than when it was fed ad libitum. Cox et al. ('58) reported that the development of atherosclerosis in the monkey was enhanced by feeding the animals once daily. Okey et al. ('60) observed that meal-eating female rats had higher serum cholesterol levels than nibblers eating the same cholesterol-rich diet. The results for the rabbits fed a sucrose-containing diet are essentially equivalent to those reported by Cohn for the chicken; however, if lactose was added to the diet, the mode of eating the diet was of little consequence to serum and liver cholesterol concentrations and atherogenesis.

SUMMARY

1. Rabbits were fed sucrose- or lactose-containing diets and cholesterol varying from trace amounts to 0.5% of the diet for 8 to 13 weeks. The maximal difference in cholesterol metabolism and atherosclerosis between the groups fed sucrose or lactose was observed when 0.35% of cholesterol was added to the diet. Essentially no difference in serum and liver cholesterol values and atherosclerosis was obtained when 0.5% of cholesterol was added to the sucrose- and lactose-containing diets.

2. The addition of 0.5 or 1% of succinylsulfathiazole (SST) to a 0.35% cholesterol-sucrose-containing diet fed to male rabbits for 8 weeks resulted in serum and hepatic cholesterol levels and atherogenesis comparable to that of the lactose-supplemented 0.35% cholesterol-containing diet. The supplementation of both SST and lactose in the diet produced essen-

[5] Broitman, S. A., D. Kinnear, L. S. Gottlieb, A. Bezman, J. J. Vitale and N. Zamchek 1959 Effect of neomycin suppression of intestinal flora and dietary magnesium on the hypercholesteremia and valvular sudanophilia induced by cholesterol and cholate diets. Federation Proc., *18:* 471 (abstract).

[6] Cohn, C., R. Pick and L. N. Katz 1959 Effect of rate of ingestion of diet ("meal eating" vs. "nibbling") on atherogenesis in chickens. Circulation, *20:* 969 (abstract).

tially the same response in the rabbit as that obtained with either component alone added to the diet.

3. During an 8-week experimental period, the restriction of the feeding pattern of rabbits given a 0.35% cholesterol-sucrose-containing diet for two or three one-hour intervals daily resulted in higher serum and liver cholesterol values and degree of atherosclerosis than the corresponding values of the ad libitum-fed controls. This effect was not observed if the diet contained 29.35% of lactose in place of sucrose. Sucrose- or lactose-containing diets consisting of either a trace or 0.7% of cholesterol were fed to rabbits on a daily alternation schedule for 8 weeks. Serum and liver cholesterol content and atherogenesis was comparable to that observed in rabbits fed the corresponding carbohydrate diets containing 0.35% of cholesterol continuously. The serum cholesterol values of the sucrose-alternate-fed groups, however, were higher than the corresponding values for the sucrose-0.35% cholesterol-fed rabbits.

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Lactose Diets and Cholesterol Metabolism

III. INHIBITION OF CHOLESTEROL BIOSYNTHESIS FROM ACETATE-1-C^{14} AND MEVALONATE-2-C^{14} BY LACTOSE OR SUCCINYLSULFATHIAZOLE-FEEDING IN THE RAT[1,2]

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The absorption of dietary cholesterol was observed to be increased by the concurrent feeding of lactose to rats (Wells and Cooper, '58) and rabbits (Wells and Anderson, '59). The ingestion of lactose was associated with an increase in the excretion of bile acids from the bile duct of cannulated rats (Wells et al., '60). Further support for a relationship between dietary lactose and bile acid metabolism was presented by Portman ('60) who reported that the half-life of isotopic cholic acid in rats fed lactose was longer than that of comparable rats fed sucrose. The bile acid pool size of the lactose-fed rats was calculated to be greater than that of the sucrose-fed controls.

Dietary cholic acid inhibited the biosynthesis of liver cholesterol from acetate-1-C^{14} (Beher and Baker, '59) by blocking some early stages of the process. Whether the cholic acid acts directly on the enzymatic steps between acetate and cholesterol or whether the inhibition is produced secondarily by the elevated liver cholesterol concentration resulting from feeding cholic acid is not clear. In view of the increased liver bile acid pool size reported to result from feeding lactose, it seemed worthwhile to study the incorporation of labeled acetate and mevalonate into rat liver and intestinal cholesterol. The present study indicated that the addition of 40% of lactose to the diet of the rat resulted in the inhibition of cholesterol synthesis from acetate-1-C^{14} in the liver but not in the small intestine. A parallel inhibition of acetate incorporation into liver sterols was also observed when the basal diet was supplemented with 1% of succinylsulfathiazole[3] (SST). In contrast, these dietary changes had little effect on the incorporation of mevalonate-2-C^{14} in either rat liver or intestine.

METHODS AND EXPERIMENTAL

Three experimental series were conducted in which either acetate-1-C^{14} [4] or mevalonate-2-C^{14} [5] served as the cholesterol precursor in the rat. Each series consisted of three dietary groups: diet A, sucrose control, was composed of (per cent): sucrose, 62.8; casein, 18; Wesson ('32) salts, 4; cottonseed oil, 15; choline chloride, 0.1; vitamin mixture, 0.1; and ample supplements of α-tocopheryl acetate in cod liver oil. Diets B and C were identical to diet A except that 40% of lactose and 1% of SST, respectively, were added at the expense of sucrose. Each group consisted of 8 male rats of the Holtzman strain (125 to 150 gm) housed in individual cages and fed the respective diets for 5 days, ad libitum. At this time, the animals of series 1 and 2 were each injected intraperitoneally with 50 μc of sodium acetate-1-C^{14} dissolved in 0.25 ml of saline. The third series was conducted exactly as the

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[2] A preliminary report of this study was presented in part before the American Society of Biological Chemists, Chicago, April 15, 1960: Wells, W. W., S. C. Anderson and R. Quan Ma 1960 The effect of lactose-containing diets on sterol metabolism in the rat. Federation Proc., *19:* 237 (abstract).
[3] Sulfasuxidine ®. Merck and Company, Rahway, New Jersey.
[4] Both radioactive precursors were obtained from Nuclear-Chicago Corporation.
[5] See footnote 4.

first two with the exception that the precursor was 1 μc of mevalonic acid-2-C^{14}-δ-lactone dissolved in 0.5 ml of 20% Tween-80.[6] The rats were placed immediately into a metabolism cage that was connected to several post absorbers containing 2 N NaOH. After one hour, the animals were decapitated. The liver and intestine were quickly removed, washed and frozen until analyzed.

TISSUE ANALYSIS

Individual liver or intestine samples were weighed and saponified with 8.5 ml per gm of 20% KOH in 85% ethanol under nitrogen for two hours. The saponified mixture was extracted with diethyl ether, the ether extract washed thoroughly with water and transferred to a suitable volumetric flask (50- or 100-ml) with acetone. Appropriate aliquots were pipetted onto planchets for determination of total nonsaponifiable fraction radioactivity.[7] Additional aliquots were removed, the solvent evaporated under a stream of nitrogen and 2 ml of acetone:absolute ethanol (1:1) were added. The sterols (chiefly cholesterol) were precipitated with digitonin as described in the Sperry-Webb ('50) procedure. For the determination of radioactivity, one aliquot was filtered onto a Whatman no. 540 filter paper and counted as above. Another sample was analyzed for mass by colorimetric analysis of the Lieberman-Burchard reaction, Moore and Baumann ('52).

RESULTS

Liver cholesterol biosynthesis. In both series, the incorporation of acetate-1-C^{14} into the nonsaponifiable and cholesterol fractions of rat liver was significantly less in either the lactose- or SST-fed rats than in the corresponding sucrose-fed controls (table 1, nonsaponifiable fraction, e.g., series 1, 79,040 count/min vs. 23,590 count/min and 29,320 count/min for sucrose-, lactose- and SST-fed animals, respectively). The relative amount of radioactivity in the cholesterol fraction of the livers was analogous to that in the nonsaponifiable fraction. Thus, the specific

[6] Polyoxyethylene sorbitan monooleate, Atlas Powder Company.

[7] Gas flow thin end window counter, Tracerlab Inc. Corrections were made for self-absorption when necessary.

TABLE 1

Effect of dietary lactose and SST[1] on the incorporation of acetate-1-C^{14} or mevalonate-2-C^{14} into the cholesterol of rat liver

Group[2]	Diet[3]	Total average nonsaponifiable fraction-C^{14}	Total cholesterol-X-C^{14}	
			Weight	Specific activity
		count/min.	*mg*	*count/min./mg*
Series 1 (acetate)				
A	Sucrose	79,040	14.7 ± 1.2[4]	544 ± 249[4]
B	Lactose	23,590	18.4 ± 2.8	169 ± 45
C	SST	29,320	15.1 ± 3.2	280 ± 144
Series 2 (acetate)				
A	Sucrose	56,900	17.9 ± 2.5	539 ± 307
B	Lactose	11,080	18.6 ± 2.1	169 ± 80
C	SST	20,040	18.8 ± 3.7	196 ± 111
Series 3 (mevalonate)				
A	Sucrose	53,290	21.6 ± 3.0	1,521 ± 418
B	Lactose	52,360	29.5 ± 5.7	908 ± 440
C	SST	42,190	19.8 ± 2.5	1,114 ± 221

[1] Succinylsulfathiazole.

[2] Rats in series 1 and 2 received 50 μg of acetate-1-C^{14} in 0.25 ml of saline intraperitoneally. Rats in series 3 received 1 μc of mevalonic acid-δ-lactone-2-C^{14} in 0.5 ml of 20% Tween-80 by the same route. Each series consisted of 8 male rats (125 to 150 gm) of the Holtzman strain.

[3] See text for description of diet composition.

[4] Standard deviation, $\sigma = \sqrt{\Sigma X^2 - \frac{(\Sigma X)^2}{N}} / N - 1$.

activity of liver cholesterol for the sucrose-, lactose- and SST-fed rats was 544 ± 249 count/min/mg, 169 ± 45 count/min/mg and 280 ± 144 count/min/mg, respectively (table 1). When mevalonate-2-C^{14} was the cholesterol precursor, only minor variations were observed in the recovery of the isotope in the liver nonsaponifiable fraction (table 1, series 3, 53,290 count/min and 52,360 count/min vs. 42,190 count/min for the sucrose-, lactose- and SST-fed rats, respectively. Inspection of the appropriate specific activities reveals that only moderate inhibition of radioactive mevalonate incorporation into liver cholesterol occurred (table 1, sucrose-fed, 1521 ± 418 count/min/mg vs. lactose-fed, 908 ± 440 count/min/mg and SST-fed, 1114 ± 221 count/min/mg).

Intestinal cholesterol biosynthesis. In contrast with the observations in the liver, no changes were noted in the incorporation of acetate-1-C^{14} into either the total nonsaponifiable fraction or the cholesterol of the intestine when the basal sucrose diet was supplemented with lactose or SST (table 2, e.g., series 1, for groups A, B, and C, the total nonsaponifiable fraction-C^{14} was 76,590 count/min, 73,850 count/min and 71,610 count/min and the specific activity of cholesterol was 2045 ± 845 count/min/mg, 2,107 ± 911 count/min/mg and 1913 ± 428 count/min/mg, respectively). When acetate-1-C^{14} was the precursor, the specific activity of the intestinal cholesterol was over three times that of the liver cholesterol from the same animals (e.g., series 1, group A, 2045 ± 845 count/min/mg vs. 544 ± 249 count/min/mg). But, when mevalonate-2-C^{14} was the cholesterol precursor, an average of 40.2% of the injected radioactivity was located in the liver after one hour while only ca. 2.0% of the C^{14} was found in the intestine. Furthermore, dietary alterations had essentially no influence on the extent of incorporation of mevalonate into intestinal cholesterol (table 2, series 3, sucrose, lactose and SST were 81 ± 37, 75 ± 33, and 98 ± 35 count/min/mg respectively).

DISCUSSION

Dietary lactose and SST may be added to a growing list of agents, which, under certain conditions, contribute to the inhibition of hepatic cholesterol biosynthesis from acetate in the rat (Kritchevsky and Staple, '60). In the present studies, the

TABLE 2

Effect of dietary lactose and SST[1] on the incorporation of acetate-1-C^{14} or mevalonate-2-C^{14} into the cholesterol of rat small intestine

Group[2]	Diet[3]	Total average nonsaponifiable fraction-C^{14}	Total cholesterol-X-C^{14}	
			Weight	Specific activity
		count/min.	*mg*	*count/min./mg*
Series 1 (acetate)				
A	Sucrose	76,590	13.8 ± 2.8[4]	2,045 ± 845[4]
B	Lactose	73,850	12.8 ± 1.8	2,107 ± 911
C	SST	71,610	12.6 ± 2.7	1,913 ± 428
Series 2 (acetate)				
A	Sucrose	62,140	15.5 ± 1.1	1,369 ± 282
B	Lactose	51,185	13.7 ± 1.8	1,286 ± 392
C	SST	47,955	14.4 ± 1.6	1,771 ± 443
Series 3 (mevalonate)				
A	Sucrose	1,917	13.5 ± 2.3	81 ± 37
B	Lactose	2,108	12.4 ± 0.9	75 ± 33
C	SST	3,147	16.2 ± 2.3	98 ± 35

[1] Succinylsulfathiazole.
[2] Rats in series 1 and 2 received 50 μc of acetate-1-C^{14} in 0.25 ml of saline intraperitoneally. Rats in series 3 received 1 μc of mevalonic acid-δ-lactone-2-C^{14} in 0.5 ml of 20% Tween-80 by the same route. Each series consisted of 8 male rats (125 to 150 gm) of the Holtzman strain.
[3] See text for description of diet composition.
[4] Standard deviation, see footnote 4, table 1.

magnitude of the inhibition of hepatic cholesterol from acetate-1-C^{14} resulting from consumption of lactose and SST, namely 69 and 49%, respectively, closely resembles that reported as a consequence of cholic acid feeding (ca. 65% inhibition, Beher and Baker, '59). The action of dietary lactose and SST on liver cholesterol biosynthesis may be attributed to the elevated enterohepatic bile acid pool size observed in rats fed these supplements (Portman, '60). In contrast with the findings in the liver, the failure to observe an inhibition of intestinal cholesterol synthesis from either acetate or mevalonate by feeding lactose or SST may indicate that other factors such as the short life span of intestinal epithelial cells (Leblond and Stevens, '48; Wells et al., '55) are more influential in controlling the rate of sterol synthesis in this tissue.

The smaller inhibition of hepatic cholesterol biosynthesis from mevalonate-2-C^{14} in the present study (30 to 26%) is also comparable to that inhibition noted by Beher and Baker ('59) as a result of feeding cholic acid to rats (25% inhibition). The high yield of liver radioactive cholesterol from mevalonate-2-C^{14} agrees with the original work by Tavormina et al. ('56). The liver, a prominent member of the reticulo-endothelial system, appears to have a much greater affinity for the nonpolar mevalonic acid-δ-lactone-2-C^{14} than the small intestine. This observation is consistent with the known ability of the liver to remove nonpolar substances or foreign bodies from the circulation (Friedman et al., '54).

SUMMARY

Male rats were fed diets containing 40% of lactose or 1% of succinylsulfathiazole (SST) for 5 days. In two experiments hepatic cholesterol biosynthesis from acetate-1-C^{14} was inhibited by 69% for the lactose-fed rats and between 49 to 64% for the rats fed SST when compared with sucrose-fed controls. When mevalonic acid-δ-lactone-2-C^{14} was used as the liver cholesterol precursor, inhibitions of only 30 and 26% for lactose and SST-fed rats, respectively, were observed. Cholesterol biosynthesis in the small intestine from both precursors was not affected by the alterations of the diet. The magnitude of the incorporation of acetate-1-C^{14} into the small intestine cholesterol was over three times that observed in the liver cholesterol. In contrast, the amount of radioactive cholesterol in the liver derived from mevalonic-δ-lactone-2-C^{14} was ca. 19 times greater than that isolated from the intestine.

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Effect of Dietary Epoxyoleic Acid Upon Rats[1]

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Although epoxy fatty acids have been known for some time to occur as a product of catalytic autoxidation of unsaturated fatty acids (Ellis, '36) it was not until recently that Gunstone ('54) described their natural occurrence in plants. Epoxy acids, previously known as oxido-acids, are characterized by having an oxirane group (–CH–O–CH–) in the fatty acid chain. This group reacts readily with both organic and inorganic acids and care must be taken to avoid prolonged exposure to acids during its isolation from natural sources. Exposure to strong acid in the course of the usual procedure for preparation of fatty acids converts the epoxy acid to hydroxy acids. Therefore the high contents of hydroxy acids reported formerly for some seed oils were largely artefacts produced from the epoxy acids present.

Since Gunstone ('54) described the occurrence of *cis* 12:13 epoxyoleic acid or vernolic acid (II)[4] in the seed oil of *Vernonia anthelmintica* which has presumed anthelmintic properties, various reports have appeared in the literature showing its presence in other seed oils (Bharucha and Gunstone, '56; Chisholm and Hopkins. '57; Hopkins and Chisholm, '59). Three other naturally occurring epoxy acids have been described lately, the structural isomer of vernolic acid or *cis* 9:10 epoxy octadec-12-enoic acid (III) (Smith et al., '60), *cis* 15:16 epoxylinoleic acid (IV) (Gunstone and Morris, '59) and *cis* 9:10 epoxystearic acid (I) (Chisholm and Hopkins, '59) which has been found also in the spore oils of some plant rusts (Tullach and Ledingham, '60). In all epoxy acids that have so far been described, the oxirane ring occurs in the same position as would otherwise be occupied by a double bond in common naturally occurring unsaturated fatty acids (table 1). Hence the suggestion was made that epoxy acids may play a role in the biogenesis of unsaturated acids (Ellis, '36; Morris et al., '61) or conversely be metabolic products of the latter (Smith et al., '60). The relationships in structure between common unsaturated acids and epoxy acids found in nature are given in table 1.

The present study of the effect of feeding *Vernonia* oil, which contains about 69% of 12, 13 epoxyoleic acid, to the rat was undertaken in an effort to ascertain whether dietary epoxy acids are incorporated into animal tissue and what effects these acids may have on the animal.

EXPERIMENTAL

The experiments summarized here were performed in two parts. A preliminary exploration of the problem (part 1) was undertaken to determine whether epoxy acids could survive the digestive tract and appear in animal tissues when administered in the diet. When this was qualitatively demonstrated, the second part of the experiment was undertaken to describe the phenomenon quantitatively and systematically.

Experiment 1. In the preliminary exploration 10 male albino rats weighing about 200 gm each were divided into two groups. Both groups received diets containing (in per cent) oil, 10; casein, 16; sucrose, 64; α-cellulose, 4; salt mixture, 4; and 1% of a casein-choline chloride mixture providing 0.115% of choline in the final

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[4] The Roman numerals refer to the epoxy acids in table 1.

TABLE 1

	Epoxy acids	Unsaturated acids	
	Formula	Formula	Common name
I	$CH_3-(CH_2)_7-\underset{O}{CH-CH}-(CH_2)_7-COOH$	$CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH$	oleic acid
II	$CH_3-(CH_2)_4-\underset{O}{CH-CH}-CH_2-CH=CH-(CH_2)_7-COOH$	$CH_3-(CH_2)_4-CH=CH-CH_2-CH=CH-(CH_2)_7-COOH$	linoleic acid
III	$CH_3-(CH_2)_4-CH=CH-CH_2-\underset{O}{CH-CH}-(CH_2)_7-COOH$	$CH_3-(CH_2)_4-CH=CH-CH_2-CH=CH-(CH_2)_7-COOH$	linoleic acid
IV	$CH_3-CH_2-\underset{O}{CH-CH}-CH_2-CH=CH-CH_2-CH=CH-(CH_2)_7-COOH$	$CH_3-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-(CH_2)_7-COOH$	linolenic acid

food mixture,[5] and the other necessary vitamins and food factors (Aaes-Jørgensen and Holman, '58). One group received 10% of *Vernonia* oil and the other, 10% of corn oil. After 10 to 15 days on this dietary regimen, the animals were decapitated. Tissue lipids were extracted in a Waring Blendor with chloroform-methanol (2:1),[6] saponified with 7 to 10% ethanolic KOH, and the unsaponifiable material removed by washing the hydrolysate several times with light petroleum ether. The aqueous solution of the soaps was then carefully neutralized with 2 N HCl and immediately extracted with ether to prevent the cleavage of the epoxide groups. After drying over anhydrous sodium sulfate, the fatty acids were converted to their methyl esters with diazomethane and chromatographed on thin layers of silica gel G (Merck) (Stahl, '58; Mangold and Malins, '60; Malins and Mangold, '60; Morris et al., '61) spread on glass plates. The eluting solvent used was diethyl ether and light petroleum ether (15:85 v/v). The extraction, saponification, methylation, and chromatography were conducted within 24 hours and, when practicable, under an atmosphere of nitrogen to avoid oxidation. The methyl esters from some of the animals were subsequently treated with anhydrous ethereal HCl to convert the epoxy acids to the chlorohydrins and rechromatographed on plates to detect possible chlorohydrins. The near infrared spectra of esters or esters treated with HCl were measured using a Beckman DK-2 recording spectrophotometer with fused silica cells of 1 cm path length, and the appearance of hydroxyl absorption at 2,795 mμ after treatment with HCl was observed as a measure of the original epoxy acid content (Morris et al., '61; Morris and Holman, '61).

Two rats fed *Vernonia* oil and two fed corn oil were killed after 10 days and the viscera, excluding the gastrointestinal tract, were examined for the presence of epoxy acids. The remaining three rats from each group were killed after 15 days and several tissues from each were ex-

[5] One per cent used of a mixture made by adding 13 gm of choline chloride to 100 gm of casein.

[6] Folch, J., M. Lees and G. H. Sloane-Stanley 1954 A simple method for preparation of total pure lipide extracts from brain. Federation Proc., *13*: 209 (abstract).

amined for their epoxy content. The treatments and analyses performed on various samples from this preliminary and exploratory experiment are listed in table 2.

Experiment 2. The second portion of the experiment was designed to find morphologic changes that might be produced by feeding vernolic acid and to examine its effect upon tissue fatty acid. Accordingly 24 male weanling rats of the Sprague-Dawley strain were divided into two groups of 12 animals each. Both groups received diets similar in composition to those described above. One was prepared with 10% of *Vernonia* seed oil and the other with 10% of olive oil. By gas-liquid chromatography and near-infrared spectrometry the fatty acid composition of the

TABLE 2

Analyses performed in experiment 1 to detect epoxy fatty acids in tissues of rats fed Vernonia oil or corn oil

Dietary fat	Days	Rat no.	Sample	Thin-layer chromatography		Near infrared analysis	
				Epoxy	Chlorhydrin after HCl	Hydroxy	Hydroxy after HCl
Vernonia oil	10	1	Viscera	+	+	−	+
			Gastric content	+ + +			
			Feces	+			
	10	3	Viscera	+	+	−	+
			Gastric content	+ + +			
			Feces	+			
	15	5	Liver	+			
			Kidney	+			
			Heart	+			
			Brain	±			
			Gastric content	+ + + +			
			Feces	+			
	15	7	Epididymal fat	+		−	+
			Liver	+		−	−
			Kidney	+		−	−
			Heart	+		−	−
			Brain	−		±	±
	15	9	Epididymal fat	+			
			Liver	+			
			Kidney	+			
			Heart	+			
			Brain	±			
Corn oil	10	2	Viscera	−	−		
			Gastric content	−			
			Feces	−			
	10	4	Viscera	−			
			Gastric content	−			
			Feces	−			
	15	6	Liver	−			
			Kidney	−			
			Heart	−			
			Brain	−			
	15	8	Epididymal fat	−		−	−
			Liver	−		−	−
			Kidney	−		−	−
			Heart	−		−	−
			Brain	−		−	−
	15	10	Epididymal fat	−			
			Liver	−			
			Kidney	−			
			Heart	−			
			Brain	−			

Vernonia seed oil was:[7] 16:0, 21%; 18:0, 0.9%; 18:1, 1.8%; 18:2, 10.5%; epoxyoleate, 69.3%; and hydroxy acids, 14.4%. The olive oil consisted of: 16:0, 10.2%; 16:1, 0.9%; 18:0, 2.4%; 18:1, 81.1%; 18:2, 4.5%; and 20:0, 0.8%. The animals fed olive oil were pair-fed, each being offered and each consuming an amount of food equal to that eaten by its mate in the *Vernonia* group the day before. The weights of all rats were recorded daily.

The animals were maintained with these diets for 28 days, killed by ether anesthesia and autopsied. Weights of livers, hearts, kidneys and epididymal fat bodies were recorded, and sections were taken from these tissues as well as from spleens, pancreases, skeletal muscle, testes and adrenals. The tissues were fixed in Zenker-formol and 10% formalin. Sections from these tissues were stained with hematoxylin and eosin. Oil red O stain for lipid was applied to paraffin and frozen sections of livers and kidneys. Epididymal fat bodies and portions of livers were quickly immersed in normal saline and frozen for chemical analyses. Determinations of the polyunsaturated acid content of livers were made by alkaline isomerization (Holman and Hayes, '58). The epididymal adipose tissue of 5 rats in the *Vernonia* group and 6 rats in the olive oil group were extracted, saponified, carefully acidified and methylated with diazomethane. Because of the possibility of alteration of the oxirane ring during gas-liquid chromatography, the epoxy fatty acid contents of these esters were determined in aliquots of the samples by near-infrared spectrometry after conversion to the corresponding chlorohydrin (Morris and Holman, '61). The remainder of the samples were analyzed by gas chromatography, and the compositions expressed as area per cent.

RESULTS AND DISCUSSION

Experiment 1. In two rats fed *Vernonia* oil, whose total visceral lipids were examined, a spot was present which migrated with methyl 12, 13 epoxyoleate standard on a thin-layer chromatograph (fig. 1). No

[7] First number indicates chain length and the number following the colon indicates number of double bonds.

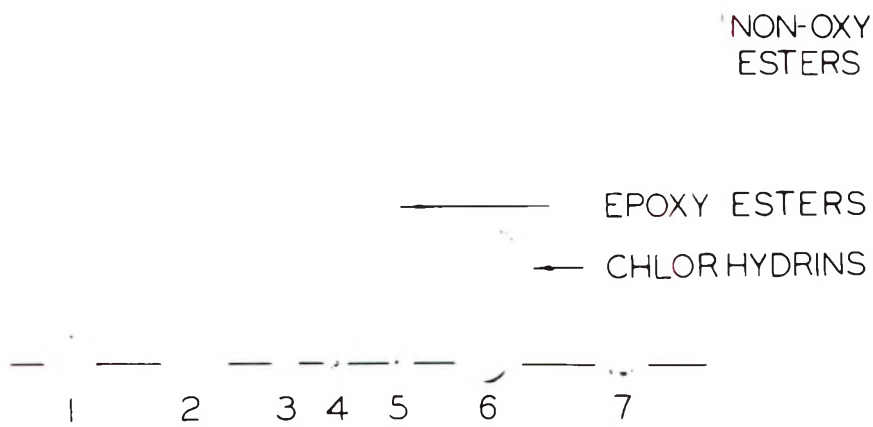

Fig. 1 Demonstration of the presence of epoxy esters in the methyl esters of fatty acids derived from tissue of rats fed *Vernonia* oil for 10 days. (1) Esters of tissue fatty acids from control rat; (2) esters of tissue fatty acids from rats fed *Vernonia* oil; (3) methyl esters of the *Vernonia* oil sample fed to rats; (4) purified methyl epoxyoleate; (5) chlorhydrins from purified methyl epoxyoleate; (6) esters of tissue fatty acids from rat fed *Vernonia* oil after treatment with HCl; (7) esters of tissue fatty acids from control rat after treatment with HCl. Spots 1, 2, 6 and 7 are grossly overloaded with respect to non-oxygenated esters to show the absence of epoxy esters in the control rat.

maxima at 2.795 μ (hydroxy groups) were noted in the near-infrared spectra. After treatment of the fatty acid esters with dry ethereal HCl, the spot attributed to epoxy ester was absent and one attributed to chlorohydrin appeared. This treatment also generated an absorption maximum at 2.795 μ (hydroxyl) in the near infrared spectrum, confirming the presence of epoxyoleate in the lipids of the viscera.

These and other qualitative tests for the presence of epoxy acids in lipids of various tissues are presented in table 2. It is clear that epoxy acids survive contact with HCl in the stomach and that they appear in both tissues and feces of rats fed *Vernonia* oil. The presence of epoxy acids in the brains of these animals is equivocal. Epoxy acids were not demonstrated in any samples or tissues from rats fed corn oil, suggesting that epoxy acids are not formed from linoleate by intestinal bacteria or by tissue metabolism in any detectable amount. Gastric contents of rats fed *Vernonia* oil contained relatively more epoxy acid than did feces, indicating disappearance during transit through the gastrointestinal tract. Present evidence does not allow decision whether this is due to absorption alone or is complicated by bacterial action.

Experiment 2. The growth curves of the rats that had been maintained with diets containing the *Vernonia* oil or olive oil showed very similar patterns, and the differences in the weight gain of the two groups was not significant at the end of the experiment. Although the kidneys, livers, hearts and epididymal fat bodies of the animals of the olive oil group tended to weigh more than those of the *Vernonia* oil group, the difference was not significant.

Structural lesions were absent in all organs studied but stainable fat (oil red O treated frozen sections) was observed in some organs. Livers from animals in both groups contained a slight-to-moderate amount of lipid droplets ranging from 1 to 2 to 8 to 10 per hepatic cell. The fat appeared most prominently in the peripheral zones of the hepatic lobules adjacent to the portal areas. In livers from those animals fed olive oil, lipid droplets were more frequent than in livers of animals fed the *Vernonia* oil. In sections of kidneys of the olive oil group, fat was prominent in proximal convoluted tubules and the difference in this respect between the two groups was more definite than for their livers. Whereas very little stainable lipid could be demonstrated in the sections from the animals fed *Vernonia* oil, a small but significant amount of oil red O stainable material was present in proximal tubules of the kidneys of the rats from the olive oil group. The fat was present in the form of droplets about the size of nucleoli, and was characteristically present between nucleus and that aspect of the cell towards the basement membrane. Not all proximal tubules contained stainable lipid, but when present, it was observed in several adjacent sections of tubules separated by others in which it was absent. Oil red O stainable material (ceroid) was not noted in paraffin sections of either livers or kidneys.

In table 3 are summarized the analyses of selected lipids from animals of experiment 2. Polyunsaturated fatty acid contents of livers were determined by alkaline isomerization. Similar analysis of pure epoxyoleate indicated apparent diene and tetraene contents of 24.7 and 16.5%. Therefore, the actual diene and triene values recorded for the liver samples should be lower because the liver lipids contained some epoxyoleate. Analyses for individual fatty acids in the epididymal fat bodies were determined by gas chromatography, except for epoxy acids which were determined by near infrared spectra.

In this relatively short-term experiment, the diet was well tolerated by the animals, and in the rats fed the diet high in epoxyoleic acid, no morphologic or biochemical lesions characteristic of a deficiency in essential fatty acids could be demonstrated. In the adult rats fed the *Vernonia* seed oil for 10 to 15 days the epoxy fatty acid was noted to make up about 2% of the total fatty acids in the adipose tissue, whereas the levels in the liver, heart, kidneys and brain ranged from 1 to 0.2% or less. In weanling rats fed the seed oil for 28 days the level had risen to a mean of 14.1%. Whether the animal is able to utilize the stored epoxyoleate as a source of energy or of essential fatty acid cannot be ascertained at this stage. Similarly no conclusions can be drawn whether the epoxy-

TABLE 3

Composition of the fatty acids from liver and epididymal fat from rats fed Vernonia oil and olive oil[1]

	Fatty acid	Vernonia oil group	Olive oil group
Liver		6 rats	6 rats
	Dienoic	212.6 ±21	99.6 ± 3.7
	Trienoic	80.4 ± 9.2	154.7 ±11.4
	Tetraenoic	377.5 ±27	234.9 ±13
	Pentaenoic	77.6 ± 5.9	41.4 ± 2.2
	Hexaenoic	61.6 ± 4.9	73.5 ± 5.1
	Trienoic:tetraenoic ratio	0.21± 0.02	0.65± 0.15
Epididymal fat		5 rats	6 rats
	Palmitic	29.0 ± 3.3	21.5 ± 1.1
	Palmitoleic	9.6 ± 1.6	6.1 ± 0.95
	Stearic	3.3 ± 1.2	2.6 ± 0.6
	Oleic	36.7 ± 3.7	65.8 ± 0.7
	Linoleic	4.7 ± 1.4	2.0 ± 0.6
	Epoxyoleic	14.1 ± 1.1	0

[1] Data for liver is expressed as mg/100 gm of tissue, and for epididymal fat as area percentage in a gas-liquid chromatogram of the methyl esters.

oleate might have a deleterious effect on general body metabolism if it were allowed to reach higher concentrations in the tissues by long-term feeding experiments.

The composition of liver fatty acids of the group fed *Vernonia* oil indicated no essential fatty acid deficiency. Although epoxyoleic acid was fed at a level of approximately 7% of the diet, no interference with the utilization of linoleate (1% of the diet) was apparent. The triene:tetraene ratio in the liver fatty acids of rats fed *Vernonia* oil was low indicating a normal essential fatty acid status (Holman, '60; Hill et al., '61). On the other hand, the present data do not allow evaluation of possible substitution of epoxyoleate for linoleate, for the 1% of linoleate fed in the diet containing *Vernonia* oil was of itself adequate to induce a normal pattern of polyunsaturated fatty acids. The composition of the fat of the epididymal fat pad reflected the unique aspects of the dietary fats as might be expected. Thus, high contents of palmitate, linoleate and epoxyoleate in *Vernonia* oil were reflected by high levels of these components in the epididymal fat of rats fed this oil. Conversely, the epididymal fat of rats fed olive oil had a higher content of oleate which is characteristically high in olive oil.

The triene:tetraene ratio of the livers of the olive oil group, however, strongly suggests an early stage or low degree of essential fatty acid deficiency (Holman, '60). This possibility is supported by the presence of more stainable fat in livers and kidneys of the animals receiving olive oil in the diet (Borland and Jackson, '31; Rice and Jackson, '34; Funch et al., '57; Alfin-Slater and Bernick, '58). The level of linoleic acid supplied by the olive oil would have been adequate to induce a normal tissue pattern of polyunsaturated acids when an otherwise fat-free diet was fed, but because of the high oleic acid content of the diet, the requirement of linoleic acid in those animals may have been raised, as demonstrated recently by Dhopeshwarker and Mead ('61).

Although limited, these experiments permit conclusion that epoxyoleic acid is absorbed and deposited in tissue lipids, and that it does not interfere with the metabolism of linoleate, to which it is closely related by structure. Its possible substitution for linoleate cannot be evaluated from these experiments. No harmful effect, either upon gross or histological aspects of tissue morphology, or upon biochemical composition of tissue lipids has been observed when 10% of *Vernonia* oil was fed for one month. Thus, amounts of epoxy acids that may be encountered in natural diets are probably not deleterious to individuals consuming them for that period.

SUMMARY

In a preliminary experiment *Vernonia* oil containing 69% of epoxyoleic acid was fed to 5 adult rats as 10% of the diet. After

10 days two rats were killed and the lipids extracted from the viscera minus the gastrointestinal tract. The fatty acids of the lipids therein were converted to methyl esters and examined for epoxy acid content by thin-layer chromatography and near-infrared spectrophotometry. Epoxy acids were observed to be present. Three other adult rats were fed the same diet 15 days and epoxy acids were noted in adipose tissue, liver, kidneys and heart of these animals. No epoxy acids were demonstrable in comparable samples from rats fed 10% of corn oil in a similar diet.

The contents of the gastrointestinal tract and feces contained epoxy acids when *Vernonia* oil was fed, demonstrating that the epoxy group survived the digestive system. No epoxy acid was demonstrable in comparable samples from the rats fed corn oil.

In a second experiment 12 weanling rats were fed a diet containing 10% of *Vernonia* oil for 28 days, and 12 controls were fed a similar diet containing olive oil. Analysis of the liver lipids demonstrated that the pattern of polyunsaturated acids was normal, suggesting that epoxyoleic acid does not interfere with utilization of linoleate. Analysis of the epididymal fat revealed 14.1% of epoxyoleic acid. Thus, this unusual dietary acid survives digestion and is deposited as other acids are.

Administration of *Vernonia* oil in the diet of rats showed no adverse effect upon gross or microscopic anatomy of the animals. Thus, it is not toxic in the level fed (7% of the diet) for the period of 28 days.

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Amprolium

V. STUDIES ON THIAMINE DEFICIENCY IN LAYING CHICKENS AND THEIR EGGS[1]

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Studies concerning the effect of thiamine deficiency on reproduction in chickens (Ellis et al., '33) provided only partial quantitative information on this vitamin deficiency. These authors showed that the composition of the diet can influence thiamine levels in yolk, indicated that chicks from hens fed diets low in thiamine die from polyneuritis soon after hatch, and confirmed the observation (Chick and Roscoe, '29) that egg white contains little or no thiamine. Other than this, information concerning thiamine deficiency in the hen is limited to the knowledge that light breed chicks (White Leghorns) have a greater resistance to thiamine deficiency than heavy breeds (Nichita and Iftimesco, '34; Lamoreux and Hutt, '39). This was attributed to a higher thiamine concentration in the yolks of light breeds by Scrimshaw et al. ('45), whose observation was later substantiated by others (Mayfield et al., '55; Howes and Hutt, '56).

Amprolium[2] [1-(4-amino-2-*n*-propyl-5-pyrimidinylmethyl)-2-picolinium chloride·hydrochloride] has anti-thiamine activity (Rogers et al., '60; Ott et al., '60). When fed to White Leghorn hens at feed concentrations in excess of 700 ppm for three weeks a decrease occurs in feed intake, egg production, hatchability and chick viability at hatch (Polin et al., '61).

The data presented in this report will show that amprolium, per se, was not toxic at the high levels fed, but adversely influenced reproduction by its anti-thiamine effect.

EXPERIMENTAL PROCEDURES

Data on feed intake, egg production, hatchability and chick viability at hatch were obtained according to procedures previously reported (Polin et al., '61). White Leghorn hens in their first year of production were kept in single cages of three-deck batteries in a constant-temperature room (21°C). Fertile eggs were obtained from the hens by use of artificial insemination with semen from roosters fed a commercial breeder ration (5.8 ppm of thiamine by thiochrome assay). The hens received this diet ad libitum except in the experiments on pair-feeding. Either amprolium or thiamine hydrochloride (USP) or both were incorporated into the breeder ration at the concentrations desired.

Experiments were 4 weeks in duration, except those involving hens pair-fed with medicated birds. During the first week, control data were obtained on each group which contained 4 or 5 hens randomly assigned to a prospective treatment. Because reproductive capacity may vary widely among groups of randomly-selected birds, data accumulated during the third week on treatments (4th week of experiment) were compared to results obtained during the control week and reported as "% of control." This equated any change caused by treatment to initial values of 100%. A nonmedicated group of hens was included in each experiment. All percentage values were converted to arcsin transformation (Snedecor, '56) before comparisons or statistical analyses were performed.

Hens were injected subcutaneously with emulsions prepared by mixing 22 ml of aqueous thiamine solution with 2.75 ml of sesame oil and 0.25 ml of Triton X-45.[3] The emulsion was used to prolong absorption of the vitamin. Fresh solutions were

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[2] Coccidiostat from Merck and Co., Inc., Rahway, New Jersey.

[3] Obtainable from Rohm and Haas, Philadelphia.

prepared weekly and stored under refrigeration. All of the hens received placebo injections of emulsion during the control week.

Embryonating eggs, incubated for 8 days, were injected with thiamine in sterile phosphate-sodium chloride buffer solution at pH 7.8 (Gortner, '49) or with the buffer alone. The vitamin was weighed from the stock bottle and transferred to sterile vials with a minimum of exposure to air. The eggs were swabbed at the larger end with 95% ethanol and drilled with an ethanol-washed 1/8 inch dental bit. Solutions were injected into the albumen with a sterile 5.0-ml hypodermic syringe and a no. 24 gauge, one-inch needle. The hole in the shell was covered with cellophane tape, sealed with paraffin, and the eggs returned to the incubator.

Yolk samples consisting of 3 to 4 pooled yolks were collected during the third week on test diets and analyzed for free thiamine and amprolium. Free thiamine was determined by the thiochrome procedure (Snell and Snell, '54) and amprolium by the method previously described (Polin et al., '61).

Yolk samples of 15 gm were weighed on tared aluminum squares, and transferred to test tubes containing 15 ml of 18% trichloroacetic acid. After thorough mixing of the contents, the tubes were allowed to stand 10 minutes, centrifuged, and the supernatants poured off through washed cotton plugs into clean tubes. One milliliter aliquots of the filtered solutions were used for the determination of free thiamine. After the development of thiochrome, it was extracted into 3 ml of isobutanol; 2 ml of the extract were transferred to a small test tube containing 0.5 ml of absolute ethanol and mixed. The solution was read in an Aminco Bowman spectrophotofluorometer using an activation wave length of 375 mμ and fluorescence wave length of 465 mμ. One milliliter aliquots of 9% trichloroacetic acid, containing zero to 1 μg per ml of thiamine, were treated exactly as yolk filtrates, and provided blank and standard fluorescence readings. When amprolium was present in the yolks, drug standards were also run, and all samples including yolks, thiamine and amprolium standards were read in the fluorometer at the wave lengths for *both* thiamine (activation 375 mμ; fluorescence 465 mμ) and amprolium (activation 400 mμ; fluorescence 510 mμ). Thiamine concentrations in the yolks were calculated by means of the following equation:

$$T = \frac{(R_A - b_A)F_{AT} - (R_T - b_T)F_{AA}}{F_{TA}F_{AT} - F_{TT}F_{AA}} \quad (1)$$

where R represents fluorometer readings of yolk samples, b represents readings of blanks (i.e., trichloroacetic acid). Single subscripts are read as follows: A, "at the amprolium wave lengths," T, "at the thiamine wave lengths." F's are standard factors; e.g., F_{TA} is the factor for thiamine at the amprolium wave lengths, F_{AT} is the factor for amprolium at the thiamine wave lengths, etc.

These factors are calculated as follows:

$$F_{AA} = \frac{S_{AA} - b_A}{C_A}, \quad F_{AT} = \frac{S_{AT} - b_T}{C_A}$$

$$F_{TT} = \frac{S_{TT} - b_T}{C_T}, \quad F_{TA} = \frac{S_{TA} - b_A}{C_T}$$

where S_{AA} reads "fluorometer reading for standard amprolium (i.e., in trichloroacetic acid) at the amprolium wave lengths," C_A is the concentration of amprolium, μg/ml, in the standard solution, b_A has the same meaning as above, etc.

F_{AT} is very small, and the product $F_{TA}F_{AT}$ is much less than $F_{TT}F_{AA}$, so that no appreciable error is introduced by omitting $F_{TA}F_{AT}$ in the denominator of (1), which then becomes:

$$T = \frac{(R_T - b_T)F_{AA} - (R_A - b_A)F_{AT}}{F_{TT}F_{AA}} \quad (2)$$

When little amprolium is present, (2) reduces further to

$$T = \frac{R_T - b_T}{F_{TT}} \quad (3)$$

By this procedure, 1 μg of thiamine added per gram of yolk was recovered 100% [control yolk 2.02 μg per gm ± 0.014 (SD); with added thiamine 3.02 μg per gm ± 0.063]; in the presence of 3 μg per gm of amprolium, recovery of thiamine was 97.6% (with added thiamine and amprolium, found 3.00 μg per gm ± 0.120).

It is possible by means of a similar equation, i.e.,

$$A = \frac{(R_A - b_A)F_{TT} - (R_T - b_T)F_{TA}}{F_{AA}F_{TT} - F_{AT}F_{TA}} \quad (4)$$

to calculate amprolium concentrations in the presence of thiamine. However, since thiamine interferes more with the determi-

nation of amprolium than is the reverse case, the absolute method for amprolium (Polin et al., '61) is preferred.

RESULTS

Feeding hens a diet containing 4,000 ppm of amprolium resulted in declines of both feed intake and egg reproduction (fig. 1). Feed intake decreased after about 4 days on medication, but egg production was not influenced until about the 11th day on medication.

1. *Oral thiamine vs. oral amprolium.* Feed intake was 32 and 12% of control during the third week hens were fed diets containing 4,000 and 8,000 ppm of amprolium, respectively (table 1). Thiamine added at 4 to 8 ppm to the breeder ration counteracted to some extent the decrease in feed intake. Ten parts per million of the vitamin were needed to completely counteract the effect on feed intake by 4,000 ppm of amprolium, whereas 100 ppm of thiamine almost returned feed intake to normal in birds fed 8,000 ppm of amprolium (table 1).

Egg production was improved by adding thiamine to rations containing amprolium. It was normal or almost normal when 50 ppm of thiamine were added to counteract 4,000 and 8,000 ppm of amprolium. Hatchability was returned to control values when at least 10 ppm of the vitamin were added to counteract the drug.

Yolk concentrations of amprolium increased when thiamine was added to rations containing amprolium at 4,000 and 8,000 ppm (table 1). The diets containing 100 ppm of added thiamine, however, were no more effective in increasing yolk concentrations of amprolium than those with the added 2 ppm, although there was almost a threefold difference in food intake. Free thiamine concentrations in the yolk, which were as low as 0.3 ppm in some of the groups fed amprolium with no added thiamine, were normal or almost normal when 100 ppm of thiamine were added to the amprolium diets.

At amprolium levels of 10,000 and 20,000 ppm, the adverse effects on feed intake and reproductive performance were

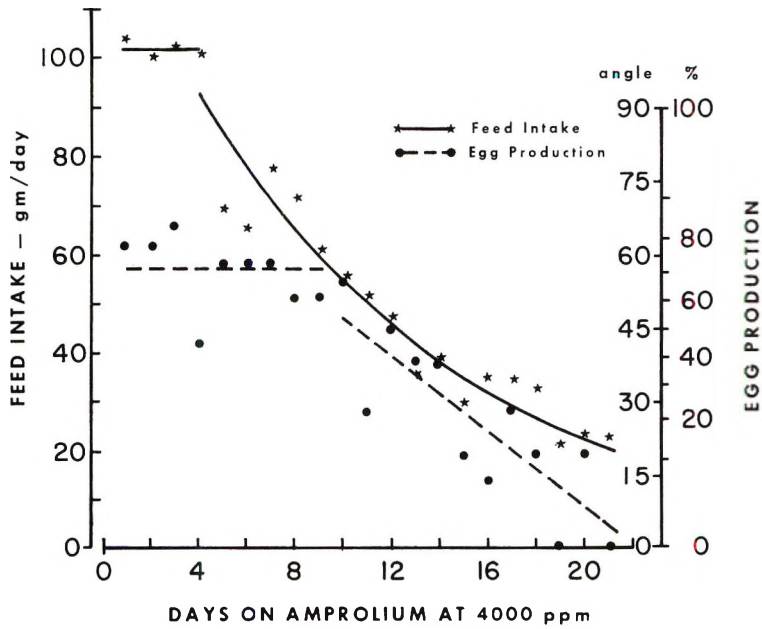

Fig. 1 The effect of feeding amprolium at 4,000 ppm for 21 days on the feed intake and egg production of hens. The parabola describing feed intake is based on the arithmetic plot of the equation $Y = 2.119 - 0.0376X$, where X = days on amprolium and Y = log of feed intake. The equation for egg production is $Y = 86.67 - 3.95X$, where X = days on amprolium and Y = angle from arcsin transformation of per cent egg production.

TABLE 1

Effect of adding thiamine to the diet on the adverse effects of amprolium on feed intake and reproduction and on the yolk concentrations of amprolium and free thiamine

Thiamine added to diet — ppm	Feed intake % of control		Egg production % of control		Hatchabilty % of control		Yolk amprolium ppm		Yolk free thiamine — ppm	
	Amprolium in diet — ppm		Amprolium in diet — ppm		Amprolium in diet — ppm		Amprolium in diet — ppm		Amprolium in diet — ppm	
	4000	8000	4000	8000	4000	8000	4000	8000	4000	8000
0	(2)[1] 32	(2) 12	(2) 21	(2) 0	(26/29 vs. 0/0)[2] —	(43/46 vs. 0/0) —	6.1	19.5	0.4	0.3
2	(1) 37	(1) 18	(1) 0	(1) 0	(24/26 vs. 0/2) 0	(17/20 vs. 0/0) —	14.0	23.9	0.5	0.2
4	(1) 43	(1) 31	(1) 52	(1) 0	(20/24 vs. 4/9) 63[3]	(19/24 vs. 0/7) 0	11.1	25.7	0.6	0.2
8	(1) 75	(1) 67	(1) 65	(1) 53	(24/27 vs. 5/9) 68	(13/15 vs. 1/6) 35	11.4	—	0.5	0.4
10	(3) 108	(1) 54	(1) 79	(1) 52	(35/38 vs. 25/28) 96	(14/17 vs. 3/4) 92	9.1	29.4	1.4	0.8
50	(1) 98	(1) 77	(1) 89	(1) 96	(17/19 vs. 16/19) 93	(13/14 vs. 12/10) 121	10.5	21.3	2.7	1.7
100	(1) 94	(1) 83	(1) 86	(1) 95	(14/18 vs. 10/14) 93	(18/20 vs. 16/20) 80	11.1	26.9	3.7	2.8
Control mean	gm/hen/day (24)[4] 112 ± 3		% production (24) 77 ± 5		% hatch (18) 87 ± 6		0		(15) 3.6[5] ± 0.14	

[1] No. pens of 4 hens each.

[2] $\frac{\text{No. hatch}}{\text{No. fertile}}$ in control period vs. $\frac{\text{no. hatch}}{\text{no. fertile}}$ in experimental period.

[3] Based on arcsin transformation of percentages.

[4] Number of control values with mean ± standard error of the mean.

[5] Based on data from this and other experiments.

TABLE 2

Effect on feed intake and reproduction of laying hens by diets containing high concentrations of both amprolium and added thiamine

Added to diet — ppm		No. of pens	Feed intake % of control		Egg production % of control		Hatchability % of control		Yolk concentration — ppm			
Amprolium	Thiamine								Amprolium		Thiamine (free)	
0	1000	1[1]	105 / —	105	113 / —	113	(7/13 vs. 8/13)[2]	109[3]	0		25.5	
10,000	1000	2	102 / 107	105	114 / 118	116	(24/28 vs. 27/29)	110	31.4 / 22.5	27.0	12.9 / 15.2	14.1
20,000	1000	2	95 / 90	93	122 / 85	104	(29/33 vs. 24/30)	91	57.9 / 38.9	48.4	10.1 / 13.6	11.9
Control mean		—	gm/hen/day (7)[4] 105 ± 4		% production (7) 64 ± 7		% hatch (7) 84 ± 6		0		(15) 3.6[5] ± 0.14	

[1] Four hens per pen.

[2] $\frac{\text{No. hatch}}{\text{No. fertile}}$ in control period vs. $\frac{\text{no. hatch}}{\text{no. fertile}}$ in experimental period.

[3] Based on arcsin transformation of percentage values.

[4] Number of control values with mean ± standard error of the mean.

[5] Based on data from this and other experiments.

TABLE 3

Feed intake, egg production, hatchability and yolk concentrations of amprolium and free thiamine of hens receiving amprolium in the diet and thiamine parenterally

Treatment		Feed intake % of control		Egg production % of control		Hatchability % of control		Weak and dead chicks at hatch	Yolk concentration — ppm			
Amprolium in diet — ppm	Thiamine subcutaneously								Amprolium		Thiamine (free)	
	μg/day							%				
4000	0	13[1] / 53	33	25 / 27	26	(35/39 vs. 0/3)[2]	0	(0)[3] —	7.9 / 6.2	7.1	0.3 / 0.3	0.3
4000	25	60 / 94	77	32 / 82	57	(36/43 vs. 5/13)	58	(5) 40	10.5 / 8.9	9.7	0.7 / 0.6	0.7
4000	50	62 / 117	90	58 / 85	72	(39/45 vs. 20/25)	91	(20) 25	20.5 / 12.1	16.3	0.3 / 0.6	0.5
4000	100	88 / 122	105	78 / 92	85	(37/42 vs. 28/28)	124	(28) 11	11.7 / 10.8	11.3	1.0 / 0.6	0.8
0	0	gm/hen/day (12)[4] 87 ± 4		% production (12) 78 ± 4		% hatch (12) 86 ± 6		(22) 0	0		4.5 / 3.2	3.9

[1] Each value represents 4 hens per pen.

[2] $\frac{\text{No. hatch}}{\text{No. fertile}}$ from control period vs. $\frac{\text{no. hatch}}{\text{no. fertile}}$ from experimental period.

[3] Number of chicks hatched.

[4] Number of control values with mean ± standard error.

prevented by the addition of thiamine to the ration (table 2). Furthermore, it was noted that hatchability was not influenced although amprolium was observed in the yolk at approximately 48 ppm (table 2). Also, free thiamine concentrations in the yolk were lower when both amprolium and thiamine were added than when only supplemental thiamine was used (table 2).

2. *Parenteral thiamine vs. oral amprolium.* The adverse effect on feed intake and reproduction from feeding amprolium can also be counteracted by injecting thiamine subcutaneously into hens. This is the more effective route of administration. As shown in table 3, 50 to 100 µg of thiamine per hen injected daily for three weeks prevented the adverse effects of the diet containing 4,000 ppm of amprolium. Comparable results were obtained when 1,000 µg of thiamine were ingested daily, an amount supplied by the diet containing 10 ppm of the vitamin (table 1). The increased chick viability at hatch, which was described in an earlier report (Polin et al., '61), was not completely counteracted by the highest parenteral dosage used. Presumably, the low yolk thiamine concentrations associated with the treatments (table 3) may have accounted for this.

3. *Thiamine injection into eggs vs. embryo mortality caused by feeding amprolium.* A hatch of 88% was obtained in eggs from nonmedicated hens as compared with a value of 56% of eggs from hens fed a ration containing 2,000 ppm of amprolium (table 4). Manipulation of the eggs in the injection experiments resulted in lower hatches, and it was these values that were used in comparing the results from thiamine injection into eggs. As shown in table 4, thiamine at 0.1 to 10.0 mg per embryonating egg had no effect on the hatch of control eggs or on the viability of the chicks that hatched. On the other hand, thiamine injected into the eggs from medicated hens improved the hatch from 29% to an average value of 46% for all medicated eggs injected with thiamine and markedly reduced the number of weak and dead chicks at hatch.

4. *Pair-feeding experiments.* The regression curve for egg production of hens pair-fed to those fed a diet containing 4,000 ppm of amprolium was almost identical to the one obtained from feeding the drug ad lib (fig. 2).

The percentage of normal chicks (total chicks hatched less weak or dead chicks at time of hatch) from the fertile eggs of hens receiving amprolium, or pair-fed with the medicated birds was similar to the percentage of normal chicks in control hatches during the first week of the experiment (table 5). Hatches from the treated hens during the second and third weeks, however, were markedly below normal, particularly those of the group receiving amprolium (table 5). In the latter case, chicks from eggs obtained during the third week on medication died soon after hatching (table 6). No weak or dead chicks were found in the pair-fed or ad libitum fed groups (table 6). Control eggs showed greatest mortality during the late stage of incubation, 18.2%, rather than during the first 7 days of incubation, 4.5%. Embryo mortality in the eggs from hens fed amprolium was increased during

TABLE 4

Hatchability and chick viability at hatch after injecting thiamine into 8-day embryonating eggs from hens fed amprolium at 2000 ppm in the diet

Egg injection	Hatchability				Chicks weak or dead at hatch			
	Amprolium in hen diet: None		2000 ppm		Amprolium in hen diet: None		2000 ppm	
		%		%		%		%
No. injection	(32)[1]	88	(25)	56	(28)[2]	0	(14)	35
Buffer solution, 0.2 ml	(43)	65	(41)	29	(28)	0	(12)	33
Thiamine, 0.1 mg	(20)	55	(36)	44	(11)	0	(16)	0
Thiamine, 0.5 mg	(10)	40	(19)	37	(4)	0	(7)	0
Thiamine, 1.0 mg	(36)	72	(41)	54	(26)	0	(22)	5
Thiamine, 10.0 mg	(10)	40	(10)	40	(4)	0	(4)	0
		59		46				

[1] Number of fertile eggs injected.
[2] Number of chicks hatched.

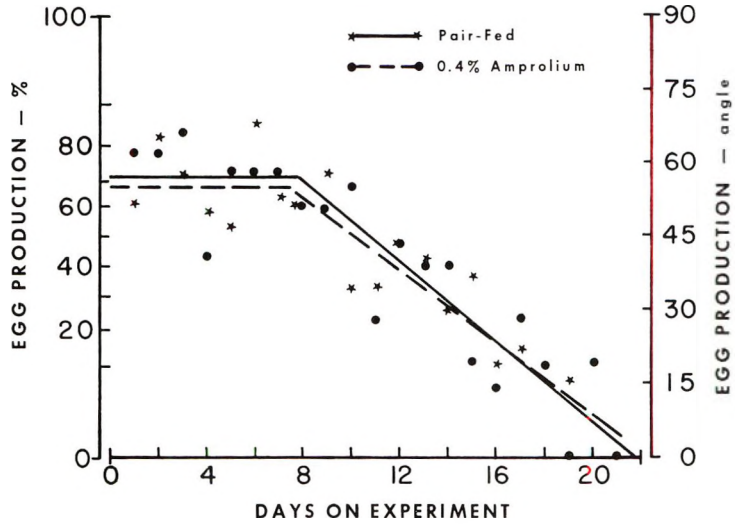

Fig. 2 Egg production during 21 days that hens were fed amprolium at 4,000 ppm or pair-fed to those receiving medicated diets. The regression equation of egg production for pair-fed hens is $Y = 82.95 - 3.72X$, and that for the medicated birds is $Y = 86.67 - 3.95X$, where X = number of days on experiment, and Y = angle from arcsin transformation of per cent egg production.

TABLE 5

Effect on hatchability (normal chicks from fertile eggs) of eggs from hens pair-fed to those fed a diet containing 4000 ppm of amprolium

Treatment		Normal chicks from fertile eggs[1] Time on experiment		
Medication	Feeding method	Week 1	Week 2	Week 3
		%	%	%
1 None	Ad libitum (control)	(40)[2] 80.0	(32) 75.0	(39) 74.4
2 Amprolium, 4000 ppm	Ad libitum	(78) 85.9	(48) 12.5	(9) 0
3 None	Pair-fed to group 2	(108) 83.3	(72) 61.1	(15) 40.0

[1] Total chicks hatched less number of chicks weak and dead.
[2] Number of fertile eggs.

TABLE 6

Embryo mortality during incubation and at hatch resulting from pair-feeding hens to those fed a diet containing 4000 ppm of amprolium

Stage of incubation	On experiment Week no.	Embryo and chick mortality		
		Treatment		
		Ad lib. (control)	Amprolium, 4000 ppm	Pair-fed
		%	%	%
Days 0–7	1	(40)[1] 2.5	(78) 5.1	(108) 4.6
	2	(32) 6.3 — 4.5	(48) 29.2	(72) 23.6
	3	(38) 5.3	(9) 22.2	(15) 40.0
Days 8–21	1	(40) 17.5	(78) 9.0	(108) 12.0
	2	(32) 18.8 — 18.2	(48) 29.2	(72) 15.3
	3	(38) 18.4	(9) 44.4	(15) 20.0
At hatch	1	(40) 0	(78) 1.3	(108) 0
	2	(32) 0 — 0	(48) 29.2	(72) 0
	3	(38) 0	(9) 33.3	(15) 0

[1] Number of fertile eggs.

both stages of incubation. This occurred as early as the second week of the experiment. Increases in mortality of the embryos from the pair-fed hens were observed during the first 7 days of incubation, and as early as the second week of the experiment.

DISCUSSION

The data presented in this and the preceding report (Polin et al., '61) are the first to quantitatively characterize the effect of marginal and submarginal thiamine deficiency on reproduction in hens. Others (Ellis et al., '33) have characterized the deficiency syndrome, but their data lacked yolk analyses for thiamine and the corresponding relationship to hatchability or mortality data of chicks at hatch, or both. Furthermore, an advantage is gained with the use of amprolium because a specific thiamine deficiency is produced while using a practical-type breeder ration.

This report shows that the adverse effects of amprolium on feed intake, egg production, embryo development and chick viability (Polin et al., '61) are not a result of drug toxicity per se but are indeed caused by a thiamine deficiency. The lower feed intake and egg production of hens which results from feeding high levels of amprolium are reversible by supplemental treatment with thiamine, either orally or parenterally. The high percentage of embryo mortality during the late stages of incubation and the poor chick viability at the time of hatch are associated with low concentrations of yolk thiamine, and as the latter is increased by treatment of hens with thiamine the hatchability returns to normal. Also, injecting eggs from medicated hens with thiamine overcomes the effect of amprolium on embryo development and chick viability.

Hens tolerated up to 20,000 ppm of the drug in the diet without any adverse effects if sufficient thiamine was provided. The egg yolks from these hens contained about 48 ppm of amprolium but no interference with thiamine metabolism of the embryo was apparent. In an earlier report (Polin et al., '61) it was demonstrated that the hatch was significantly below normal when amprolium was fed at 2,000 ppm, yet the yolks from these hens contained as little as 4 ppm of amprolium. Thus, the tolerance of hens to amprolium (up to 20,000 ppm) depends on the amount of thiamine provided, as established in the present experiments.

The lower production of eggs caused by feeding amprolium to hens appeared to be a result of lower feed intake induced by a thiamine deficiency. This was apparent from the pair-feeding experiment.

When diets containing 2,000 ppm of amprolium are fed to hens, embryo mortality increases during the late stage of incubation and at hatch. No increase in mortality occurs during the first 7 days of incubation, and the hens laying these eggs show a reduction in feed intake of about 20% (Polin et al., '61). When diets containing 4,000 ppm of amprolium are fed, however, an increase in embryo mortality is observed during the early stages of incubation in addition to the mortality at the later stages. This early peak of embryo mortality is attributed to the marked reduction in feed intake that is characteristic of hens receiving 4,000 ppm of amprolium in the diet. It was presumably not due to a thiamine deficiency because yolks from the pair-fed hens had normal thiamine concentrations (table 7), and

TABLE 7

Yolk thiamine and amprolium concentrations in eggs collected during the third week hens were fed 4000 ppm of amprolium or pair-fed to medicated birds

Treatment	Yolk concentration	
	Amprolium	Thiamine (free)
	ppm	*ppm*
None (ad lib.)	0	(15)[1] 3.6 ± 0.14
Amprolium, 4000 ppm	(7) 7.4 ± 0.96	(7) 0.3 ± 0.14
Pair-fed to amprolium	0	(2) 2.9, 3.8 3.4

[1] Number of pooled yolk samples.

also because the mortality peak was not observed in thiamine deficient eggs from hens fed 2,000 ppm of amprolium (Polin et al., '61).

One other consideration is the fact that the lower feed intake caused by high levels of amprolium was produced by a thiamine deficiency and not a distaste for the feed. The latter was excluded as a reason when it was demonstrated that hens fed high levels of the medicant maintain a normal feed intake when injected with sufficient thiamine. This fact is most important because the inanition response by the hen to thiamine deficiency appears to be the primary symptom, and presumably occurs from depressed physiological function of brain center(s) which regulate the desire for food consumption.

SUMMARY

The coccidiostat, amprolium [1-(4-amino-2-*n*-propyl-5-pyrimidinylmethyl)-2-picolinium chloride·hydrochloride], when fed in the diet at 2,000 ppm or more to laying hens, produced lowered feed intake, decreased rate of lay, increased embryo mortality, and lowered chick viability at hatch. Associated with the subnormal hatches were markedly depressed free thiamine concentrations in the yolk, in some instances to barely detectable amounts. All of the adverse effects were counteracted by oral or parenteral administration of thiamine, which in turn, also elevated yolk thiamine concentrations. The latter route of administration was more effective in counteracting 4,000 ppm of amprolium in the diet. An injection of 50 to 100 μg of the vitamin was required as compared with the 1 to 10 mg ingested via the daily intake of 100 gm of diet containing 10 to 100 ppm of supplemental thiamine, respectively.

High concentrations of amprolium in yolks had no adverse effect on the hatch, and as much as 20,000 ppm of amprolium in the diet were tolerated by the hen provided sufficient thiamine was administered.

A reduction in food intake comparable to that caused by the feeding of amprolium produced similar losses in rate of lay. Amprolium increased embryo mortality during the first 7 days of incubation, during the later stage of incubation, and at hatch. Pair-feeding experiments revealed that inanition accounted for the higher embryo mortality during the first 7 days of incubation.

In view of the lack of toxicity of amprolium per se, and the ability of thiamine to counteract the adverse reproductive effects, the symptomatology attributed to feeding high concentrations of amprolium to laying hens is considered reflective of an induced thiamine deficiency.

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Degossypolized Cottonseed Meal as a Source of Plant Protein in Rabbit Feeds

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The sensitivity of nonruminants to toxic cottonseed meal rations has been recognized and investigated for several decades. Concerning the rabbit, Voris et al. ('40) noted intestinal stasis, cecal impaction, bloating, and death in animals fed cottonseed oil cake pellets plus an equal weight of alfalfa hay. More recent studies by Holley et al. ('55) demonstrated hemorrhage of the small intestines, lungs and brain, enlarged gall bladders, edema, anuria, and impaction of the large intestine in rabbits receiving cottonseed meals at levels of 20 and 25% of the ration. Cottonseed meal containing 0.04% of free gossypol proved to be toxic when fed as 20% of the diet, and mortality occurred in some animals where the calculated individual intake was not more than 100 mg of free gossypol. The results of these workers indicated that gossypol is absorbed slowly by rabbits and acts as a cumulative poison, the habit of coprophagy apparently increasing the sensitivity of the rabbit to this toxic substance. Many commercial milling companies are including degossypolized cottonseed meal in rabbit pellets at an undisclosed level. Apparently, it is serving as a satisfactory source of protein and, during most seasons of the year, is one of the least expensive sources of plant protein available.

The purpose of this experiment was to study the tolerance of the rabbit for degossypolized cottonseed meal and to evaluate its use as a replacement for other plant protein sources in rabbit rations.

MATERIALS AND METHODS

Design. A split-plot randomized block design was followed. Thirty mature New Zealand White females (does) were bred to 6 unrelated bucks, forming 6 half-sib blocks. The 5 does within each block were randomly assigned to one of the 5 rations which were fed throughout 4 successive litters. Where sisters were available they were assigned to different blocks and received different rations.

Feeding. Five rations were used, one control and 4 experimental (table 1). The basic stock ration in use at the U. S. Rabbit Experiment Station served both as the control and as the basis of the other 4 rations. Does received the various rations throughout pregnancy and lactation until the young were weaned. Rations were available to the young from the time they left the nest box at approximately three weeks of age, until weaning. All rations were fed free choice.

General. All animals were housed in all-metal, self-cleaning hutches equipped with automatic waterers. Does were rebred 52 to 53 days following parturition and palpated for pregnancy 12 days following breeding. They were weighed when placed on test and when each of their litters was weaned. Young were weighed, weaned and removed from experiment at 56 days of age. As far as possible, litters were equalized at 8 young each within three days following parturition. The original number in a litter was then referred to as the "number retained." Illness of does and young, and any treatments administered, were recorded. All animals that died on test were weighed and autopsied to determine cause of death. Feed consumption was determined from parturition to weaning.

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[3] See footnote 2.

[4] See footnote 1.

TABLE 1

Composition and chemical analyses[1] of rabbit rations

	Rations				
	1 (Control)	2	3	4	5
	%	%	%	%	%
Suncured alfalfa meal	40.0	40.0	40.0	40.0	40.0
Soybean meal (expeller)	18.0	15.0	13.0	11.0	9.0
Linseed meal (expeller)	4.0	4.0	4.0	4.0	4.0
Barley	18.5	18.5	18.5	18.5	18.5
Oats	4.0	4.0	4.0	4.0	4.0
Wheat mixed feed (millrun)	15.0	15.0	15.0	15.0	15.0
Salt	0.5	0.5	0.5	0.5	0.5
Degossypolized cottonseed meal[2]	0.0	3.0	5.0	7.0	9.0
Crude protein	21.74	19.76	20.62	20.18	20.63
Ether extract	3.14	3.14	3.09	2.99	2.88
Crude fiber	14.62	14.98	14.40	15.16	14.76
N. F. E.	45.16	46.80	47.14	47.13	46.59
Ash	7.74	7.50	6.96	7.04	7.13

[1] Determined on an air-dry basis.
[2] Free gossypol, 0.016%; total gossypol, 0.79%. Supplied by San Joaquin Cotton Oil Company, Los Angeles.

Analysis of data. Results were evaluated on the basis of average kindling weaning weight per litter, total litter weaning weight, mortality, and feed conversion as determined by amount of feed required to produce a pound of weight at weaning.

Total litter weaning weight and average kindling weaning weight were analyzed with number weaned and rabbit feeding days used as multiple covariates.

Examination of the means of male and female weaning weights showed no sex difference. The mean weaning weights and standard errors were 3.88 ± 0.03 pounds and 3.87 ± 0.04 pounds for the males and females, respectively. Since two litters had no average weaning weights, due to litter mortality prior to weaning, missing data were computed after Federer ('55), and the analysis of variance computed.

Feed conversion data were analyzed by analysis of covariance with feeding days as the covariate (Federer, '55), after estimating one missing plot.

Mortality data were subjected to analyses of variance of both percentage of mortality and the arcsin transformation of percentage of mortality (Federer, '55).

The analysis of variance determined the significance of block and ration differences over whole plots; and litter, block × litter and ration × litter differences over sub plots.

RESULTS AND DISCUSSION

In table 2 are shown the ration means for average kindling weaning weight per litter, total litter weaning weight, feed conversion and percentage of mortality. The analysis of variance of total litter weaning weight ration means indicated no significant differences among rations. However, the linear and quadratic responses of total litter weaning weight to additional cottonseed meal were found to be significant at the 0.05 level. No other significant ration responses were found in the analyses of average kindling weight per litter, feed conversion, percentage of mortality or the arcsin transformation of percentage of mortality.

The significant quadratic regression of unadjusted total weaning weight on percentage of cottonseed meal was caused by better growth with the 3 and 5% rations (table 2). The significant linear regression of unadjusted total weaning weight on percentage of cottonseed meal reflects an increasingly harmful effect when more than the optimal level of cottonseed meal is included in the ration. When the ration means were adjusted to a constant number weaned and a constant number of rabbit feeding days per litter a less negative linear response was noted. This would be expected since the correlation between the total litter weaning weight and number weaned was + 0.95, the correlation between total litter weaning

TABLE 2

Ration means and standard errors

Ration	Average kindling weight per litter		Total litter weaning weight		Feed conversion	Mortality	No. weaned	Rabbit feeding days
	Unadjusted	Adjusted[1]	Unadjusted	Adjusted[1]				
	pounds	*pounds*	*pounds*	*pounds*		%		
1	3.97	4.01 ± 0.08	20.06	20.78 ± 0.37	3.96	26.9	5.1	204
2	3.81	3.82 ± 0.08	22.54	20.29 ± 0.40	3.70	19.4	5.8	220
3	3.93	3.93 ± 0.07	21.24	20.58 ± 0.37	3.98	25.5	5.4	209
4	3.79	3.74 ± 0.08	19.68	18.85 ± 0.38	3.81	24.8	5.4	202
5	3.86	3.86 ± 0.09	16.61	19.63 ± 0.43	4.36	37.1	4.4	184
Standard error	0.09		1.26		0.21	4.6	0.3	13

[1] Adjusted for covariance of number rabbits weaned and rabbit feeding days. In this type of analysis standard errors will differ among rations.

weight and rabbit feeding days was + 0.78, and the correlation between number weaned and rabbit feeding days was + 0.87. Therefore, when these data are adjusted for either number weaned or rabbit feeding days they are automatically adjusted to a large extent for the other.

The correlation between average kindling weaning weight per litter and number weaned was − 0.24 and the correlation between rabbit feeding days and average kindling weaning weight per litter was − 0.48. The larger the litter size the less milk each kindling would receive, on the average. Hence, individual growth would be retarded. Likewise with more total rabbit feeding days per litter there would still be less per individual in the larger litters. Since the correlations were smaller between the independent variables and average kindling weaning weight per litter than between these same variables and total weaning weight, the covariance analysis made less adjustment in the average kindling weaning weight per litter than for total litter weaning weight. This again may be traced back indirectly to percentage of mortality, the correlation between percentage of mortality and average kindling weaning weight being + 0.10, whereas the correlation between percentage of mortality and total litter weight was − 0.50. Therefore it seems that the harmful effect, if any, of increased cottonseed meal on growth was counterbalanced to a large extent (for individual kindling weaning weight) by the beneficial effect of making available more milk to the survivors of the litter.

Although the percentage of mortality was not found to differ significantly among rations, the data in table 2 suggest that the 9% of cottonseed meal may increase the percentage of mortality and thus depress total litter weight.

Studies by Cabell and Earle ('56), Stevenson and Earle ('57), and Hale and Lyman ('57) suggest that relatively high protein levels tend to prevent the toxic effects of free gossypol. Since the crude protein levels in the rations used in these investigations approximated 20 to 22% (table 1), it is possible that gossypol tolerance and amino acid balance might be more critical using rations containing 15 to 18% of protein, such as those commonly used in commercial rabbit production. Further studies involving controlled protein levels are needed to answer this question.

SUMMARY

Degossypolized cottonseed meal was fed to 30 mature female rabbits throughout 4 successive litters, using a split plot, randomized block design. When used as a replacement for soybean oil meal, at levels of 3, 5, 7 and 9% of the rations, no significant ration responses were found in the analyses of average kindling weaning weight per litter, percentage of mortality, or feed conversion. The linear and quadratic responses of total litter weaning weight to additional cottonseed meal were significant at the 0.05 level. Indications of a depressing effect on total litter weaning weight were found at the 7 and 9% levels. Results indicate that, at levels of

3 and 5%, degossypolized cottonseed meal is a satisfactory source of plant protein in rabbit rations.

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Influence of High and Low Caloric Intakes on Fat Deficiency of Dogs[1]

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The clinical observation that the young rapidly growing human infant is likely to develop a dry and scaly skin with thickening when fed milk mixtures extremely low in fat content (Hansen et al., '58) prompted further study of the possible relationship between caloric intake and manifestations of fat deficiency. Heretofore, most studies concerning variations in calorie intake have been devoted to the effects of restricted intake on body size (McCay, '35; Jackson, '37; Schultze, '55; Berg, '60) or life span and disease (McCay et al., '43; Berg and Simms, '60) or in relation to the utilization of protein (Bosshardt et al., '48; Hegsted and Haffenreffer, '49; Rosenthal and Allison, '51; Leverton et al., '51; Rosenthal, '52; Calloway et al., '55). Berg ('60) and Berg and Simms ('60) have extended the studies concerning longevity and onset of disease in the rat in relation to food intake giving particular emphasis to the pathologic changes in various tissues. Histology of the skin was not discussed.

The purpose of the present study was to evaluate the influence of low, normal and high caloric intakes on the development of fat deficiency signs and symptoms in young puppies which subjects are known to be particularly susceptible to a dietary deficiency of linoleic acid.

MATERIALS AND METHODS

At 7 to 11 weeks of age 27 Beagle puppies were given either a diet low in fat or a control diet in which fat replaced part of the carbohydrate calories. For 20 animals, protein derived from skim milk powder and casein provided 15% of the calories and for 7 animals, 20% of the calories. In the low-fat and control diets, carbohydrate other than that furnished by the skim milk was given as sucrose. Fresh steam-distilled lard[2] which provided 15% of the calories as fat in the control diet furnished approximately 2% of the calories as linoleic acid. Minerals were given in the form of an artificial bone ash mixture and Cowgill's ('23) salt mixture. Each day's diet was supplemented with cellulose[3] and a multivitamin preparation.[4] The skim milk powder was not vitamin-free.

Sixteen puppies were fed the low-fat diet and 11 puppies served as controls. In both groups, littermates were given weighed amounts of food that were considered to be at low, normal and high caloric levels. These represented intakes of 100, 150 and 200 Cal. per kg per day from 6 weeks to 4 months of age. From 4 to 6 months of age, the intakes were decreased by 15%, providing 85, 127 and 170 Cal. per kg per day. It was not possible to decrease the intakes proportionately, thereafter, because animals in the high-calorie group refused to eat all of the food offered and those in the low-calorie group appeared too emaciated to attempt further reduction in energy intake. By this time, depending upon the amount of food consumed, pronounced effects on growth, clinical condition, histology of the skin and blood lipids were noted. In the presentation of results for the animals receiving the low, normal and high caloric intakes, these will be designated as the

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[2] Supplied through the courtesy of the Wilson Company, Oklahoma City, Oklahoma.

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

[4] Poly-Vi-Sol (Mead Johnson and Company, Evansville, Indiana) supplied daily: vitamin A, 1,250 IU; vitamin D, 250 IU; thiamine, 250 μg; riboflavin, 300 μg; niacin, 2 mg; ascorbic acid, 12 mg. Vitamin E given as *d*-α-tocopheryl acetate, 25 mg weekly.

100, 150 and 200 Cal. per kg per day groups.

All animals were free from intestinal parasites and were housed in separate metal cages in an air-conditioned room maintained at 23.5°C. For the first two weeks after weaning, young puppies were fed one-half of their rations twice daily. Thereafter, all animals were fed once daily. Care was taken to observe whether all food was consumed each day. At weekly intervals the animals were weighed before feeding.

Skin biopsies were taken from the interscapular area and dorsal surface of the thigh. For histologic examination, sections were stained with hematoxylin and eosin. Blood samples were taken from all dogs after an overnight fast. Total proteins of blood serum were determined by a modification of the Kingsley method ('42). Blood serum analyses for lipids included the amount of the total fatty acids with the distribution of the saturated and unsaturated fatty acids. Total, di- tri- and tetraenoic acids were determined by the method of Wiese and Hansen ('53) using a Beckman ratio-recording spectrophotometer for density readings of the isomerized soaps. Distribution of the saturated and unsaturated fatty acids was determined by gas-liquid chromatography using the Beckman GC-2 instrument with a 12′ diethylene glycol succinate column at a temperature of 220°C and helium as the carrier gas.

RESULTS

Effects on growth. Gain in weight and size with respect to height, length, and breadth were considered as criteria for rate of growth. In figure 1 are presented typical weight curves at two to 7 months of age for three littermates fed the control diet containing lard and for three littermates supplied with the low-fat diet at levels of 100, 150 and 200 Cal. per kg per day. On the basis of 100 Cal. per kg per day, there was essentially no change in weight for either the puppies fed the

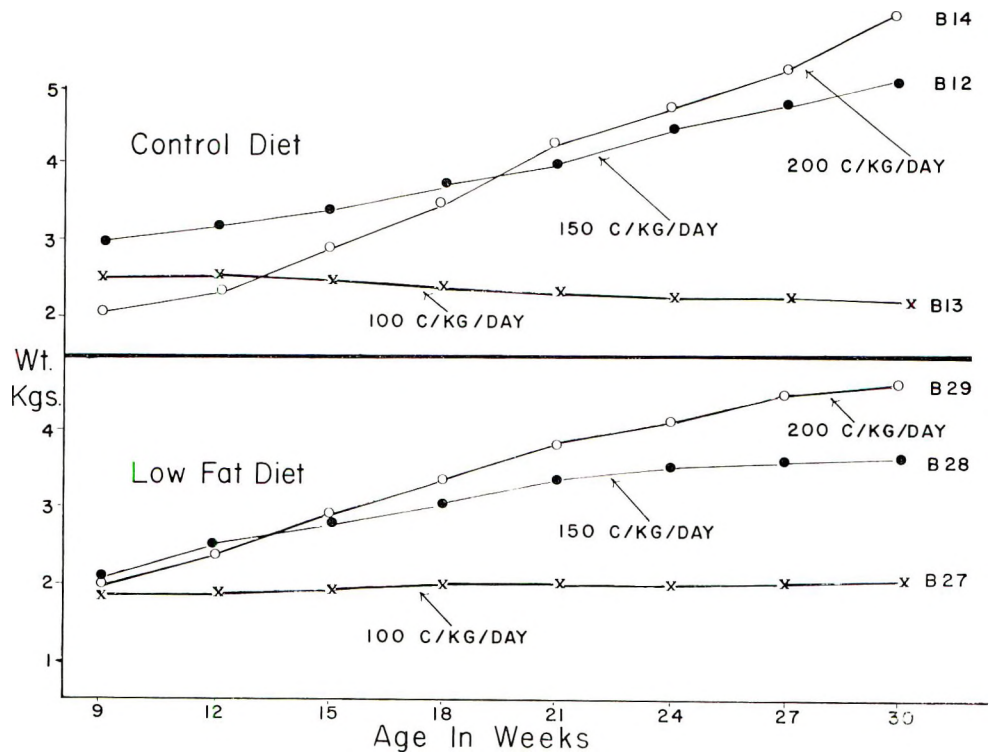

Fig. 1 Growth curves for littermates at 100, 150, 200 Cal./kg/day (2 to 7 months).

diet without fat or those that received lard. At the level of 150 Cal. per kg per day, which is generally considered adequate for young growing puppies after 10 weeks of age (Allison, '55), the rate of gain in weight was satisfactory for both groups although there was a trend for the rate of growth to level off at 6 months of age in the animals that did not receive fat. For both groups at the 200 Cal. per kg per day level, the rate of weight gain definitely was accelerated over that at 150 Cal. per kg per day. The gain in weight was somewhat greater for the animals that received fat than for those that received the low-fat diet.

Differences in the size of these 6 animals are apparent from figures 2 and 3.

Clinical effects. For the control animals receiving fat, except for variations in rate of growth, no gross clinical differences at the three caloric levels were noted. The skin remained smooth and soft and the hair glossy in appearance. In the low-fat group, however, development of fat deficiency signs definitely was related to the amount of food consumed. Puppies fed the high caloric intake began to show coarse, dry hair and desquamation on the ventral surface after two to three months and were severely deficient after 4 to 5 months (6 to 7 months of age). At a normal caloric intake (150 Cal. per kg per day) early signs of fat deficiency began to appear approximately one month later than for the littermates on the high-calorie intake. Even at 7 months of age no signs of skin involvement were evident in puppies receiving 100 Cal. per kg per day of the low-fat diet. Desquamation with coarse hair in a 6-month-old dog fed a high-calorie, low-fat diet is illustrated in figure 4.

Histologic alterations. Microscopic sections taken from the interscapular area and dorsal surface of the thigh showed similar patterns which correlated well with the gross appearance of the animals. Histologically, there was no difference between the skin of control littermates receiving 100, 150 and 200 Cal. per kg per day. However, littermates that received

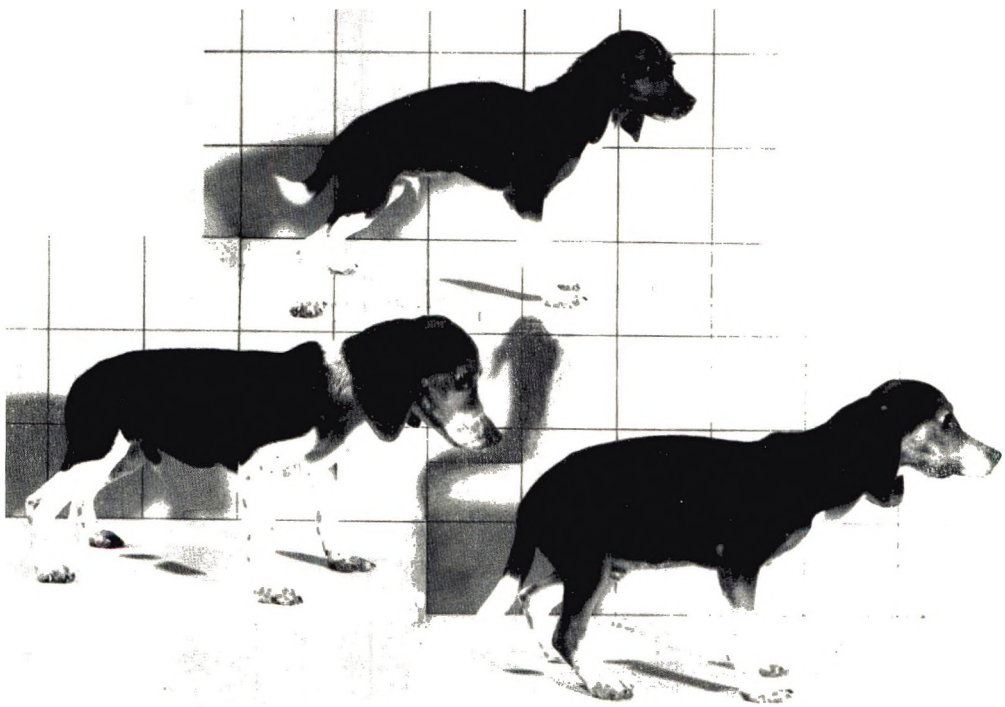

Fig. 2 Littermates fed control diet containing lard at 100, 150, 200 Cal./kg/day (top to bottom).

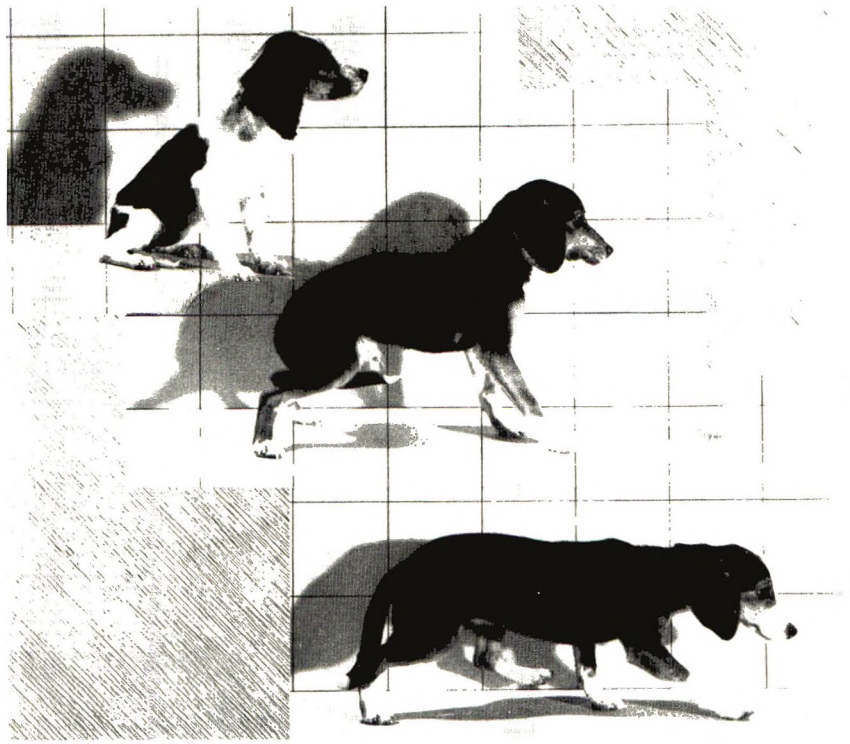

Fig. 3 Littermates fed low-fat diet at 100, 150, 200 Cal./kg/day (top to bottom).

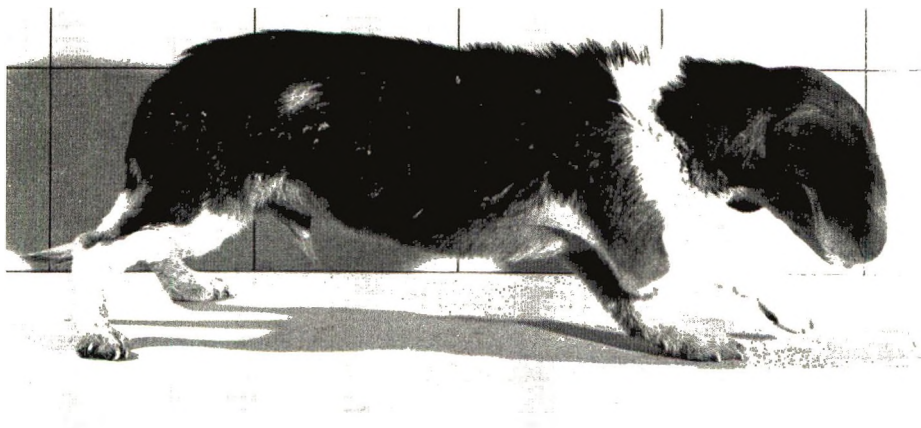

Fig. 4 Low-fat diet at 200 Cal./kg/day for 4 months.

the low-fat diet at the three caloric levels showed marked differences. At 7 months of age, the skin of puppies fed the low-fat, low-calorie diet was essentially normal in appearance, showing a thin epidermis of two to three cells in thickness with a lacelike keratin layer. On the other hand, the skin of littermates which consumed 150 or 200 Cal. per kg per day showed a thickened epidermis with deranged keratinization of the epidermal layer. The thickness of the epidermal layer varied from 4 to 6 or 10 to 12 cells depending on the severity of the fat de-

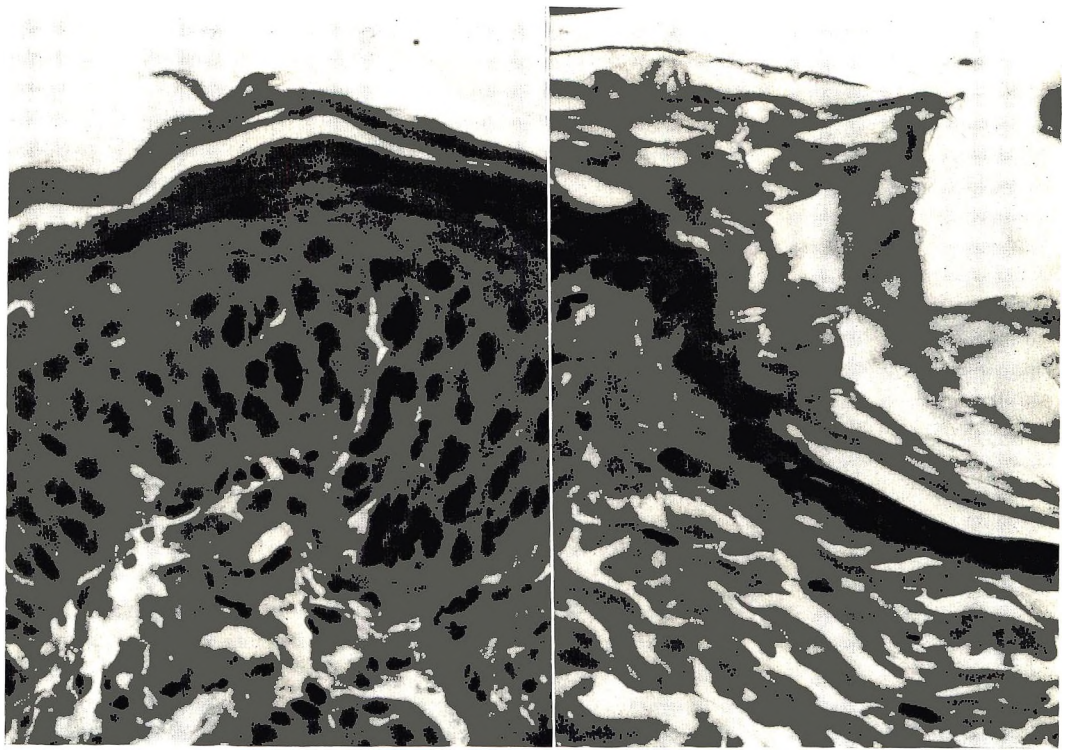

Fig. 5 Photomicrographs of skin of littermate dogs (age 6 months) fed low-fat diet at 200 Cal./kg/day (left) and 100 Cal./kg/day (right).

ficiency state. In the advanced state of deficiency, parakeratosis was evident as well as infiltration of cells in the dermis. In figure 5 are illustrated typical histologic sections of skin from littermates which had received 200 and 100 Cal. per kg per day of the low-fat diet for 4½ months (age 6½ months).

Blood serum protein. No significant differences were noted between littermates in serum levels for total protein that could be correlated with caloric intake or the fat intake. Although the puppies receiving the 100 Cal. per kg per day diets consumed only half as much protein (gm per kg per day) as their littermates at the 200 Cal. per kg per day level, the animals at the lower intake with 15% of the calories as protein were ingesting 3.7 gm per kg per day of protein which Allison ('55) has considered adequate for puppies after 10 weeks of age under laboratory conditions. The mean serum protein levels were 4.70, 4.92 and 4.94 gm per 100 ml for the low-, normal- and high-calorie groups, respectively.

Blood serum lipids. As previously reported (Wiese et al., '57) the mean total fatty acid levels were definitely lower for all the animals fed the low-fat diet than for those which received lard in the diet. In the present study, however, caloric level did not appear to influence the serum levels of the total fatty acids for either group. Graphic representation of the distribution of the principal saturated and monoene fatty acids is shown in figure 6. Each bar represents the mean value for three to 6 animals in each group. Palmitic and stearic acids were consistently lower in the serum of puppies that received the low-fat diet than in the animals that received fat. The levels for these fatty acids were quite constant for each group regardless of caloric intake. The monoene fatty acids, palmitoleic and oleic acids, however, were consistently greater in the group fed the low-fat diet than in the

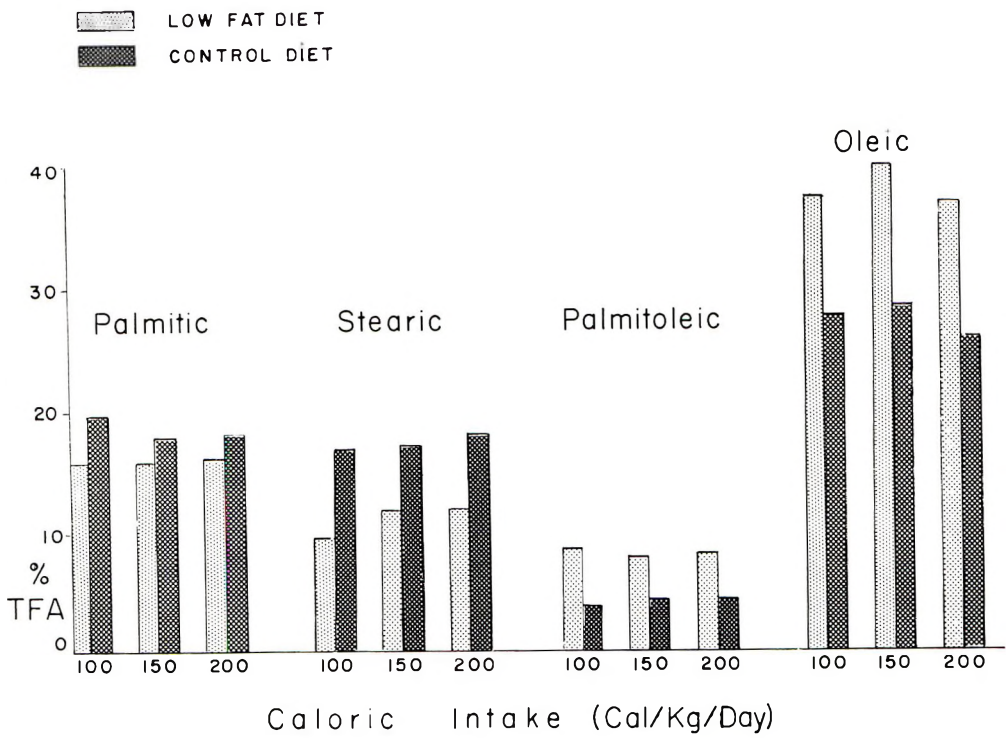

Fig. 6 Saturated and monoene fatty acids in blood serum of young Beagles (age 5 to 7 months).

control group and again were strikingly constant at the three caloric levels. In addition to the saturated and monoene fatty acids illustrated in figure 6, serum of all puppies showed small amounts (1% or less) of myristic and myristoleic acids which were slightly higher for the low-fat groups than for the controls.

Linoleic and arachidonic acids were the only di- and tetraenoic acids found by gas-liquid chromatography and are designated as such in figure 7. In some instances, particularly in the case of the animals fed the low-fat, low-calorie diet, two adjacent peaks appeared on the chromatograms which were assumed to be the two trienoic acids derived from palmitoleic and oleic acids and identified by Mead and Slaton ('56), Mead ('57) and Fulco and Mead ('59) in tissues of linoleic acid deficient rats as 7, 10, 13 and 5, 8, 11-eicosatrienoic acids. When fat deficiency signs were evident grossly at the 150 and 200 Cal. per kg per day levels, the amount of trienoic acid in the serum was greatly increased and appeared on the chromatograms as one large peak.

The amounts of the di- tri- and tetraenoic acids expressed as percentage of the total fatty acids in serum of fat-deficient puppies were related to the caloric intakes (fig. 7). Linoleic and arachidonic acids were considerably higher for the puppies that did not grow at 100 Cal. per kg per day compared with littermates at 150 and 200 Cal. per kg per day. Also appreciably less trienoic acid was synthesized by the puppies that were not gaining weight and that did not show clinical or histologic evidence of fat deficiency. For the control animals that did not grow at 100 Cal. per kg per day, likewise, the serum level for linoleic acid was definitely higher than for littermates that were gaining weight. The control low-calorie puppies did not synthesize as much trienoic or arachidonic acid as littermates receiving the higher caloric intakes. All control animals received about 2% of their calories as linoleic acid and there were no evidences of gross or histologic skin abnormalities.

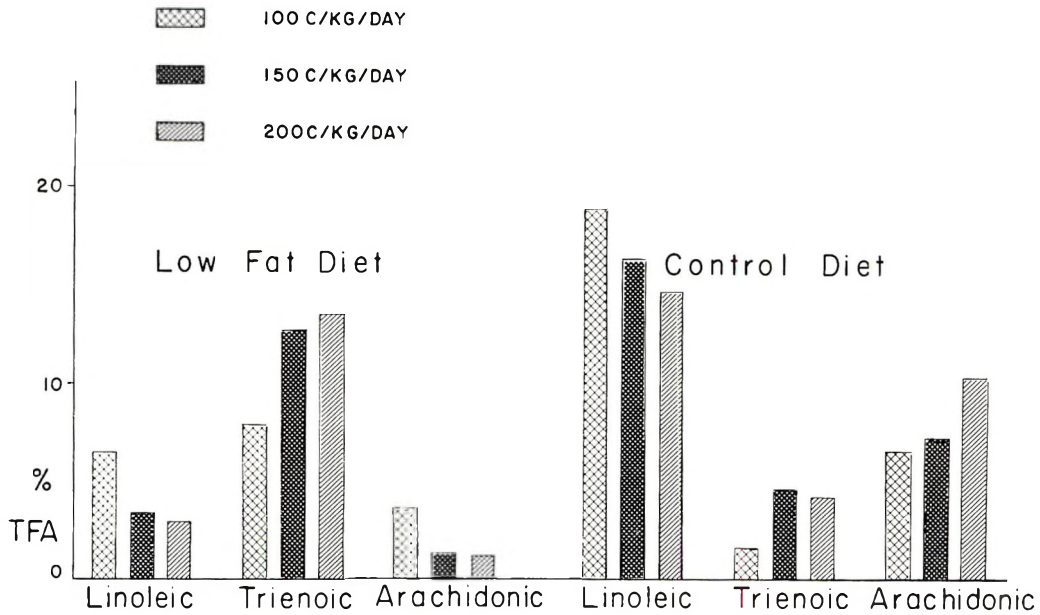

Fig. 7 Di- tri- and tetraenoic acids in blood serum of young Beagles (age 5 to 7 months).

DISCUSSION

Although skin changes typical of fat deficiency have not been reported in adult subjects, Barki and associates ('47) noted the development of fat deficiency symptoms in mature rats following ad libitum feeding of fat-free diets to animals that had lost one-half their weight as a result of restricted intakes. No symptoms of fat deficiency were noted in these rats before the ad libitum feeding began. After 66 days, however, there was spontaneous disappearance of deficiency signs which suggested to the authors some synthesis of essential fatty acid by the animals. It is not clear how much of the gain in weight of the mature rats was due to growth in the sense that new tissue was being formed and how much was due to an increase in body fat. If animals are able to synthesize small amounts of linoleic acid, which may be sufficient for maintenance of an adult animal, it is not adequate for growth. This was clearly demonstrated by the rate of development of fat deficiency signs and symptoms for Beagle puppies during the period of most rapid growth (two to 7 months of age) wherein the rate of growth was influenced by the caloric intake of the diet.

It is well known that biochemical findings for blood or tissue composition for one or more components can be extremely valuable in assessing nutritional status even though overt clinical symptomatology may not be evident. In this study a combined approach including observations on rate of growth, gross and histologic appearance of the skin and biochemical changes in the fatty acid components of blood serum was undertaken. Failure to note clinical signs of fat deficiency in the puppies that failed to grow was confirmed by the histologic examination of the skin which showed a normal structure. Distribution of unsaturated fatty acids of blood serum also correlated well with the rate of growth and the rate of development of the fat deficiency state. The observation of increased amounts of palmitoleic, oleic, and trienoic acids in the serum of puppies deprived of dietary fat compared with those in littermates receiving fat in the diet confirms the findings of Mead ('57) who demonstrated more of these fatty acids in tissues of rats maintained with diets low in fat than in control rats. Williams and Scheier ('61) also reported that these fatty acids increased in amount in the liver of young rats after the admini-

stration of pyridoxine to animals that had been deprived of both vitamin B_6 and essential fatty acids. Although the pyridoxine-supplemented rats receiving a diet low in fat did not show visible dermal symptoms of fat deficiency, they had gained in weight.

In our study, the serum levels for the di- tri- and tetraenoic acids were of particular significance in relation to the caloric intakes of both young dogs maintained on a diet low in fat and those fed a diet containing lard. Serum values for these fatty acids as determined by alkaline conjugation of the total fatty acids checked exceedingly well with those found by gas-liquid chromatography.

SUMMARY

Observations made on 27 Beagle puppies during their rapid growth period (two to 7 months of age) indicate that the rate of development of fat deficiency signs and symptoms was related directly to the rate of growth which in turn was dependent on the caloric intake of the low-fat diet.

1. Puppies that did not grow at 100 Cal. per kg per day levels with a diet very low in fat content showed neither gross nor histologic evidences of fat deficiency during a 5-month period (7 months of age). Littermates receiving 150 Cal. per kg per day of the same diet had satisfactory growth rates and in three to 4 months developed gross and histologic evidences of fat deficiency. Littermates consuming 200 Cal. per kg per day showed accelerated rates of growth and fat deficiency signs, grossly and histologically, about one month sooner than puppies at the normal intake of 150 Cal. per kg per day.

2. Blood serum levels for palmitic and stearic acids were consistently lower for all puppies fed the low-fat diet regardless of caloric intake compared with those of littermates receiving approximately 2% of their calories as linoleic acid in the form of fresh lard.

3. The monoene fatty acids, palmitoleic and oleic, were consistently higher in blood serum of the animals fed low fat than in the control group, again, regardless of caloric intake.

4. Linoleic and arachidonic acids in blood serum of dogs fed the low-fat, low-calorie diet did not decrease to the low levels observed for littermates that received normal or high caloric intakes. Also there was less trienoic acid synthesized by the puppies that failed to grow.

5. In control littermates that received dietary fat containing linoleic acid, growth response was similar to that of animals fed the low fat, at the same caloric levels but the skin and hair remained normal in appearance.

6. Linoleic and arachidonic acid levels in blood serum were significantly higher for the control than for the low-fat group receiving the same caloric intakes. There was some indication that control puppies that failed to grow utilized less linoleic acid than their rapidly growing littermates.

ACKNOWLEDGMENT

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Dental Caries in Rats Given Various Diets and Water with a Low Concentration of Fluoride[1]

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Most authors report that relatively high levels of F^- as sodium fluoride in the drinking water are required to obtain as much as 50% reduction in experimental caries in the albino rat (Hein, '55).

Rats and hamsters differ in their reaction to fluorides. Hein ('55) noted that to produce maximal inhibition of dental caries 125 ppm of F^- as sodium fluoride in the drinking water was required for albino rats, but only 12 ppm for hamsters.

Wuthier and Phillips ('59), in a study of long-time administration (approximately 90 to 360 days) of low levels of fluoride, found that 1.2 and 3.2 ppm of F^- in the drinking water had "no significant protective effect against dental caries." Wynn and Haldi ('55), desiring to establish the effect of natural water supplies or of fluoridated water, found that drinking water containing 0.7 to 1 ppm of F^- given to rats after weaning had no more protective influence on experimental dental caries in albino rats than did distilled water. McClure et al. ('59) produced smooth surface caries by a diet consisting largely of skim milk powder, cornstarch and 18% of glucose, that was inhibited by 25 and 50 ppm of F^-.

The aim of the experiments reported here was to determine whether a relatively low concentration of fluoride (3 ppm of F^-) in the form of NaF as contrasted with tap water (0.05 ± 0.01 ppm of F^-) would be effective in reducing caries in susceptible albino rats fed several types of diets. (Hawaii has a high per capita sugar consumption, a high dental caries rate, and the water supplies are low in fluorides.) It was reported in 1958 (Miller, '58) that a susceptible strain of rats developed little or no caries when fed a semi-natural diet and tap water, but that the rats showed severe caries when the diet was modified to include 17% of sugar by weight. This caries-producing diet is similar to a diet simulating a typical American diet containing 17% of sugar which was used by Zepplin et al. ('50). These workers reported that this diet caused as severe carious lesions in the cotton rat as a cariogenic diet with 67% of sucrose.

EXPERIMENTAL

The mothers of all rats received semi-natural diet 15 consisting of the following in grams: skim milk powder, 350; whole wheat flour, "fine grind," 650; cornmeal, 150; white flour (enriched), 150; brown rice flour, 150; soybean flour, 100; yeast, 30; cotttonseed oil,[2] 46; cod liver oil, 4; salt mixture, 10; and iodized salt, 10; all ground to pass a 28-mesh sieve (Miller and Schlack, '58).

Two experiments were conducted. For experiment 1, 8 litters of rats were used. Rats from 5 litters were weaned at 21 days and divided randomly into two groups of 21 rats each, so that each contained about the same number of males and females of approximately the same weights. Animals in group 1 received tap water and diet 18 (same as diet 15 except that 17% of the whole wheat flour was replaced by powdered sugar).[3] All ingredients of the experimental diets were ground to pass through a 48-mesh sieve. Rats in group 2 received the same diet but were given tap water containing NaF to supply 3 ppm of F^- in addition to the fluorine present naturally — 0.05 ± 0.01 ppm. Group 3 was made up of rats from three litters, also

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[2] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

[3] The powdered sugar used in these experiments, as well as those previously reported, was sucrose containing 3% of cornstarch.

weaned at three weeks of age but all the mothers were given fluoridated water (3 ppm of F^-) the last two weeks of pregnancy and throughout lactation. These rats were bred and the young raised during the same period as the rats in groups 1 and 2.

Because there was a definite improvement in the dental conditions of the rats given tap water with 3 ppm of F^- added, a second experiment was carried out with three groups fed different diets and given water with and without added NaF as before.

For experiment 2, 15 litters of rats weaned at 21 days were divided into 6 groups of 20 each. Groups 1a and 1b were fed diet 15 (ingredients passed through 48-mesh sieve). Groups 2a and 2b were fed diet 19 in which 12% of the whole wheat flour of diet 15 was replaced by powdered sugar,[4] and for groups 3a and 3b, diet 18 with 17% of the whole wheat flour replaced by powdered sugar. Groups 1a, 2a, and 3a received only tap water; groups 1b, 2b, and 3b received tap water with 3 ppm of F^- added in the form of NaF. The diets were of the same degree of fineness as for experiment 1.

After the rats had received the experimental diets for 100 days, all were sacrificed. The treatment of the jaws, and the technique of examining and scoring the teeth were the same as reported earlier (Miller and Schlack, '58).

The methods of statistical analyses used for these experiments were those previously reported from this laboratory (Miller and Schlack, '58). Prior to subjecting the data to evaluation by the analysis of variance the original observations were, in all instances, subjected to transformation (Snedecor, '57, pp. 314–321). The specific transformations used were $(n + 3/8)^{1/2}$ for the number (n) of defective teeth and for the number of carious areas, and the $\log_{10}(s + 1)$ for the caries scores (s).

RESULTS AND DISCUSSION

The data for experiment 1 are summarized in table 1 and for experiment 2 in table 2.

For this susceptible strain of rats the results for experiment 1 showed a highly significant reduction in dental caries when 3 ppm of F^- were added to the drinking water as judged by three criteria — number of teeth affected, number of carious areas, and extent of the carious lesions (caries score). There was no significant difference between the two groups receiving fluoride, so that in this instance providing 3 ppm of F^- during the period of tooth development was no more effective than giving it only postweaning.

Shaw and Sognnaes ('55) reported that 6 ppm of F^- had no effect in reducing caries whether given during tooth development and postweaning or only after weaning. When 25 ppm of F^- were given during tooth development a statistically significant reduction in the number and extent of carious lesions was achieved, but

[4] See footnote 3.

TABLE 1

Results of giving water with and without fluoride to rats fed a cariogenic diet (exp. 1)

Category of interest	Group 1	Group 2	Group 3
Diet no.	18	18	18
Water	tap water	tap water +3 ppm F^-	tap water +3 ppm F^-
No. of rats	21	21	21
Rats with caries	21	19	18
Carious teeth (total no.)	117	79	69
Carious teeth/rat	5.57	3.76[1]	3.28[1]
Carious areas (total)	211	130	109
Carious areas/rat	10.05	6.19[1]	5.19[1]
Carious scores (total)	338	201	167
Caries score/rat	16.10	9.57[1]	7.95[1]
Mean weight, males, gm	369	382	370
Mean weight, females, gm	244	245	230

[1] Values significantly less than for group 1 ($P < 0.01$).

TABLE 2

Results of feeding three groups of rats three different diets with and without fluoridated water (exp. 2)

Category of interest	Group 1		Group 2		Group 3	
Diet no.	15 (no sugar)		19 (12% sugar)		18 (17% sugar)	
Water	tap water	3 ppm F^-	tap water	3 ppm F^-	tap water	3 ppm F^-
No. of rats	20	20	20	20	20	20
Rats with caries	12	11	19	17	20	19
Carious teeth (total no.)	44	40	117	64	125	89
Carious teeth/rat	2.20	2.00	5.85	3.20[1]	6.25	4.45
Carious areas (total)	65	56	196	92	226	148
Carious areas/rat	3.25	2.80	9.80	4.60[1]	11.30	7.40[2]
Carious scores (total)	97	74	332	135	383	255
Caries score/rat	4.85	3.70	16.60	6.75[1]	19.15	12.75
Mean weight, males, gm	356	350	359	354	357	351
Mean weight, females, gm	228	231	225	235	247	247

[1] Values significantly less ($P < 0.01$) than for littermates receiving tap water.
[2] Value significantly less ($P < 0.05$) than for littermates receiving tap water.

the change in number of carious molars was not significant even with this relatively high level of fluoride.

In experiment 2, one may note the effect of 3 ppm of F^- in the drinking water after weaning when diets with zero, 12, and 17% of sugar were fed to littermate rats in contrast with those receiving tap water (table 2). For the semi-natural diet 15, there were some improvements in dental conditions when 3 ppm of F^- were added to the drinking water of the rats, but the results did not prove to be statistically significant using the usual three criteria. For diet 18, containing 17% of sugar by weight, there was an improvement in dental conditions when F^- was added to the water, but only for carious areas was it sufficient to be statistically significant ($P < 0.05$). In this experiment, the changes for teeth and caries score were not quite great enough to be statistically significant. Fluoride in the drinking water had the greatest influence in improving the dental conditions when the diet contained 12% of sugar (table 2). For these rats there was a significant difference ($P < 0.01$) as judged by all three criteria — number of carious teeth, number of carious areas, and the caries score.

When rats were fed a relatively good diet, little or no improvement in dental conditions resulted from furnishing water with about 3 ppm of F^-. If the diet was made highly cariogenic by the addition of 17% of sugar, water with the same amount of fluoride reduced the detrimental effect of the diet, but the dental condition was well below that of the control diet. With a smaller amount of sugar (12% by weight), a distinct improvement in dental conditions resulted when 3 ppm of F^- were added to the tap water.

Comparing only the rats receiving fluoridated water at a level of 3 ppm of F^-, the results may be summarized as follows. There was a significant difference ($P < 0.01$) in the condition of the teeth as judged by the usual three criteria between those fed the semi-natural diet (15) and the diet containing 17% of sugar (18); but the differences between the two diets containing sugar, and between diets 19 and 15 were not great enough to be statistically significant.

These results show that a low level of fluoride (3 ppm of F^-) added to the drinking water had an effect upon the teeth of rats depending upon the type of diet fed. It is possible that this would also be true for humans consuming fluoridated water, especially if the diets are high in sugar.

SUMMARY

Two groups of littermate rats susceptible to dental caries were fed a semi-natural cariogenic diet (17% of sugar by weight) after weaning with tap water and with

fluoridated water (3 ppm F^-). A third group was given the same low level of fluoride during the developmental period and also postweaning. There was a marked improvement ($P < 0.01$) in the dental conditions of both groups receiving the fluoride, but no significant difference between the two.

A semi-natural diet without sugar and two cariogenic diets (12 and 17% of sugar) were fed to 6 groups of littermate rats given water without and with added fluoride (3 ppm of F^-). Dental caries of rats fed the semi-natural diet showed little or no improvement with fluoridated water, and the highly cariogenic diet showed some improvement. For the rats given the lesser amount of sugar, there was a statistically significant ($P < 0.01$) decrease in dental caries, as judged by the number of carious teeth and of areas, as well as the caries score.

These experiments suggest that for humans, the nature of the diet might well influence the improvement that could be expected when fluoridated water is used.

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Avian Disease Virus and Nutrition Relationships

III. EFFECT OF NEWCASTLE DISEASE VIRUS ON NITROGEN RETENTION IN THE IMMATURE FOWL[1,2]

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The adverse effect of infection on nitrogen retention has been reported for malaria (Barr and DuBois, '18), pneumonia, and streptococcal infections (DuBois, '27), and other infections common to man (Coleman et al., '22; Coleman and Gephart, '15; McCann and Barr, '20; Narsinga-Rao and Gapalan, '58).

There are a number of phenomena associated with disease infection that may be related to an adverse retention of nitrogen. Studies in our laboratories (Squibb, '61) have shown that Newcastle disease virus (NDV) infection in White Leghorn cockerels increases mortality and significantly reduces feed intake. The experiments reported here were undertaken to study the relation of NDV infection to dietary nitrogen intake and retention in immature fowl.

METHODS AND RESULTS

In each of the nitrogen balance trials, day-old White Leghorn cockerels from the same source were provided water ad libitum and a stock diet containing 21% of crude protein (Squibb, '61) for a 35-day preliminary period. This interval was selected to deplete the birds of any parental immunity to the NDV. The chicks were matched on the basis of weight gains during the preliminary period and then randomly assigned to blocks; each block contained all treatments. While on balance, infected and noninfected chicks were kept in individual stainless steel wire cages in isolated air-conditioned rooms. Preparation of the NDV, inoculation procedures, confirmation of infection, and management of the chicks were those previously described (Squibb, '61).

The stock diet fed in the preliminary period was also used for the balance trials; water was provided ad libitum. Fecal collections of individual birds were made 24 hours prior to NDV inoculation and thereafter every 48 hours during a 12-day balance period. These collection intervals were chosen to coincide with incubation of the virus (2 to 3 days); active involvement (3 to 4 days); and initiation of recovery (4 days) (Squibb, '61). Total feces of each individual chick for each collection period were pooled, the nitrogen determined, and retention calculated according to procedures outlined by Sanslone and Squibb ('62). Data were analyzed according to Snedecor ('57).

Experiment 1. This experiment used a total of 70 chicks in two balance trials. In the first trial there were three treatments per block: (1) noninfected controls fed ad libitum; (2) noninfected controls with feed intake restricted to that of the NDV-infected birds; and (3) NDV-infected birds fed ad libitum. In the second trial the same three treatments were repeated, and a 4th group, which was NDV-infected and force-fed as well as given feed ad libitum, was added. Force-feeding was accomplished by preparing a slurry composed of 100 gm of the stock diet and 160 ml of water. An average of 70 gm of this mixture was placed in an infected bird's crop at intervals dictated by the emptying of the crop.

At the end of the experiment the data of each trial were combined; the data of

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the birds which survived were grouped and analyzed apart from those which had succumbed to the infection.

Nitrogen intake and retention of the NDV-inoculated birds that failed to survive the infection, and that of the noninfected controls, are presented in table 1. As would be expected, the noninfected ad libitum-fed birds, representing normal, growing chicks, remained in positive balance and showed a gradual increase in N retention. The infected birds retained more N than their respective controls during the incubation period of the virus ($< 5\%$).[3] This was followed by a rapid decline into negative balance until death occurred ($< 1\%$). When N intake of noninfected controls was restricted to that of the infected chicks fed ad libitum, N retention declined in the same manner as in the NDV-infected animals ($< 1\%$). Although force-feeding had no effect on mortality, there was a slight increase in N intake during the incubation and start of the active involvement periods over that observed in the NDV-infected birds fed ad libitum. Nitrogen retention, however, was similar to that of the other infected chicks.

The data of the NDV-inoculated chicks that survived, and the controls in the same blocks, are shown in table 2. Nitrogen retention in the feed-restricted and ad

[3] Snedecor ('57) *F* values.

TABLE 1

Experiment 1: Average nitrogen intake and retention of immature White Leghorn cockerels that failed to survive infection with Newcastle disease virus while on various feeding regimens

Treatment	Days post-inoculation						
	0	2	4	6	8	10	12
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
	Nitrogen intake						
Control, ad libitum	2.09(13)[1]	2.87(13)	3.04(13)	2.78(13)	3.28(8)[2]	—	—
Control, restricted	2.22(15)	2.65(15)	2.10(15)	0.72(15)	0.17(9)	0.03(3)	—
NDV, ad libitum	2.32(15)	2.68(15)	1.50(15)	0.25(14)	0.04(8)	0.03(2)	—
NDV, ad libitum, force-fed	2.34(7)	3.07(7)	1.72(7)	0.48(5)	—	—	—
	Nitrogen retention						
Control, ad libitum	0.86(13)	1.14(13)	1.30(13)	1.22(13)	1.28(8)	—	—
Control, restricted	0.87(15)	0.99(15)	0.44(15)	−0.38(15)	−0.92(9)	−1.47(3)	—
NDV, ad libitum	0.85(15)	1.40(15)	0.23(15)	−0.64(14)	−0.81(8)	−0.43(2)	—
NDV, ad libitum, force-fed	0.87(7)	1.52(7)	0.00(7)	−0.44(5)	—	—	—

[1] Number of chicks indicated in parentheses.
[2] Upon death of an NDV-infected chick, the noninfected mates in the same block were removed.

TABLE 2

Experiment 1: Average nitrogen intake and retention of immature White Leghorn cockerels that survived infection with Newcastle disease virus while on various feeding regimens

Treatment	No. of chicks	Days post-inoculation						
		0	2	4	6	8	10	12
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
		Nitrogen intake						
Control, ad libitum	7	2.59	3.02	3.09	3.02	3.47	3.49	3.61
Control, restricted	5	2.41	2.88	2.26	1.67	1.05	2.18	2.83
NDV, ad libitum	5	2.54	2.93	2.21	1.07	1.66	2.56	2.95
NDV, ad libitum, force-fed	3	2.55	2.63	2.23	1.32	1.04	2.34	3.13
		Nitrogen retention						
Control, ad libitum	7	0.98	1.21	1.35	1.21	1.48	1.47	1.50
Control, restricted	5	1.00	1.06	1.02	0.14	−0.16	0.65	1.17
NDV, ad libitum	5	1.17	1.60	0.76	−0.27	0.39	1.29	1.49
NDV, ad libitum, force-fed	3	0.88	1.10	0.54	0.01	0.22	1.62	2.01

libitum fed-controls followed the same pattern as that of the noninfected controls shown in table 1. Again, during the incubation period of the virus, N retention was higher in the NDV-infected chicks than in the controls, without a corresponding increase in N intake (< 5%). The initial rise was followed by decreased N retention during the active involvement stage of the NDV (< 1%). The decline, which was also shown by the feed-restricted noninfected controls, was not of the same extent as that observed in the NDV-infected birds that died (table 1). In the last two collection periods, which coincided with recovery from the infection, the increase in N retention in the infected as well as the noninfected feed-restricted chicks was equal or greater than that of the ad libitum fed-controls. Force-feeding did not increase N intake, but the same rise and fall in N retention was noted in the birds so treated as in the ad libitum-fed infected chicks during the incubation and active involvement periods of the NDV. In the course of recovery from the infection, however, N retention in these birds surpassed that of all other groups, including the noninfected ad libitum-fed controls.

Experiment 2. From the data of experiment 1, it appeared that N retention during NDV infection was more heavily influenced by lower dietary N intake due to inappetence than the disease per se. Therefore, experiment 2 was designed to observe the effect of NDV infection on N retention without the confounding of anorexia. This was accomplished by reducing the viral potency of the inoculum from 10^{-3} to 10^{-9}. Standardization of the "H" strain of NDV in this laboratory showed that this concentration of virus would produce 100% the symptoms of NDV in susceptible chicks and result in immunization without reducing feed intake. Each block contained two treatments: (1) noninfected controls; and (2) NDV-infected birds. The blocks were replicated, making a total of 10 birds per treatment. All birds were given feed and water ad libitum.

When the virulence of the infection was reduced by infecting the chicks with an inoculum of lower viral concentration, N retention was not depressed (table 3). Moreover, the N retained by the infected birds showed the same initial rise above the controls as that noted in experiment 1 (< 1%); N retention, however, remained above that of the controls until the end of the balance period.

DISCUSSION

Newcastle disease virus (NDV) infection of the immature White Leghorn cockerels in these experiments followed the same syndrome of incubation, active involvement, and initiation of recovery from the disease as that previously described (Squibb, '61).

The pattern, but not the degree, of N retention during the incubation and active involvement stages of NDV was the same for birds that survived as for those that succumbed to the infection. In each case there was an initial rise in N retention above that of the noninfected controls during the incubation phase of the virus, a phenomenon which was not related to an increased intake of dietary N. The initial rise was followed by a rapid decline in N retention during the active involvement

TABLE 3

Experiment 2: Average nitrogen intake and retention of immature White Leghorn cockerels immunized with Newcastle disease virus and fed ad libitum

Treatment	No. of chicks	Days post-inoculation						
		0	2	4	6	8	10	12
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
		Nitrogen intake						
Control	10	1.56	2.55	2.73	2.78	3.03	3.31	3.23
Infected	10	1.56	2.75	2.87	3.03	3.51	3.35	3.50
		Nitrogen retention						
Control	10	0.71	0.95	1.11	1.12	1.09	1.16	1.08
Infected	10	0.68	1.26	1.39	1.42	1.30	1.48	1.02

stage of the disease. In all cases the decline, which was greater in the birds that succumbed, was related to a depressed intake of dietary N, as shown by the data of the feed-restricted noninfected controls.

Force-feeding failed to provide a continuous increase in N intake or influence N retention during the active involvement stage of the disease. In the recovery phase, however, the infected birds on this regimen showed greater retention of N than the ad libitum fed noninfected controls.

Additional evidence that dietary N intake was the principal influence on N retention during NDV infection was demonstrated in the birds that were inoculated with an immunizing level of the virus (10^{-9}). In these chicks, dietary N intake was not depressed. Nitrogen retention showed the same initial rise observed in the groups with a greater concentration of the virus (10^{-3}). With the lower virus concentration, however, N retention remained higher than that of the controls until the recovery stage of the NDV. This phenomenon was similar to that observed by Luca and Constantinescu ('58) in children infected with poliomyelitis.

The initial rise in N retention in the infected birds was apparently the result of less N excretion via the urine, since comparison with the controls revealed similar N intake for both groups at this time. It is possible that this apparent conservation of body N is related to defense mechanisms.

SUMMARY

Newcastle disease virus (NDV) infection of immature White Leghorn cockerels on ad libitum, restricted, and force-fed regimens, increased nitrogen retention during incubation of the disease and depressed retention in the active involvement stage. The extent to which the infection influenced N retention was related to the virulence of the disease which, in turn, affected dietary N intake.

Only during the recovery stage of the disease, when dietary N intake returned to normal, did N retention approach that of the noninfected controls.

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Effect of a Magnesium-Deficient Diet on Magnesium Metabolism in Rabbits: A Study with Mg^{28} [1,2]

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The classic experiments of Orent et al. ('34) showed that bone magnesium is depleted in rats maintained with magnesium-deficient diets. Knoop et al. ('39) subsequently found that soft tissues were not appreciably depleted of magnesium even under the most severe conditions of deficiency. Duckworth and Godden ('41) suggested that under conditions of deficiency, bone magnesium supplied the needs of the soft tissues. These interpretations were based on chemical analyses of tissues for their content of magnesium.

The dynamics of magnesium transfer in tissues can best be studied by the use of a radioactive isotope of this element as a tracer, and Aikawa et al., ('59) have previously demonstrated the sensitivity of this new tool. McAleese et al. ('61) have recently used Mg^{28} to investigate the effects of a magnesium-deficient diet in lambs. The purpose of the present experiment was to study the effect of a magnesium-deficient diet on the tissue uptake of Mg^{28} and on the exchangeable magnesium content in the rabbit. This study is one phase of a project aimed at determining the various factors regulating the metabolism of magnesium.

EXPERIMENTAL

Twelve male, adult rabbits were placed in individual stainless steel metabolism cages and were given without restriction throughout the experiment tap water which contained 0.6 mEq per liter of magnesium.

The basal diet used for rats by Mackenzie and Mackenzie ('59) was supplemented by the salt mixture of Hubbell et al. ('37), except that the magnesium salts were omitted.

The composition of this diet, expressed in grams per kilogram, was as follows: vitamin-free casein, 200; sucrose, 668; lard,[3] 100; vitamin mix, 10; choline chloride, 2; and magnesium-free salt mixture, 20. Menadione, 2.5 mg and oleum percomorphum, 10 drops, were added to each kilogram of the mixture. This diet was made up fresh weekly, and was supplemented each week by 20 mg of α-tocopherol given orally with a medicine dropper. Each kilogram of this Mg-deficient diet contained 6.6 mEq of Mg; the stock diet[4] contained 172 mEq.

Mg^{28} was received as $MgCl_2$ in concentrated HCl. Magnesium was precipitated as $Mg(OH)_2$ with an excess of 1N NaOH. The precipitate was dissolved with 1N H_2SO_4, and the Mg was diluted in distilled water to a final concentration of 0.4 mEq per ml. This solution was used without sterilization.

Before the rabbits were fed each morning, the body weight, total food consumption, and 24-hour urine volume were measured in order to follow the external balance of magnesium. Since the Mg content of the tap water was small compared with its content in the Mg-deficient diet, the intake of Mg in water was not taken into consideration in the calculation of the external balance.

The exchangeable magnesium content, Mg_e was measured as follows. Each animal was given 5 ml of the Mg^{28} solution intravenously (a total of 2 mEq of Mg and 10 μc of Mg^{28}). A pooled specimen of urine was then collected from each animal over

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[2] Mg^{28} was supplied by the Brookhaven National Laboratory, Upton, Long Island, New York, on allocation from the U. S. Atomic Energy Commission.

[3] Cudahy Rex, Cudahy Packing Company, Denver, Colorado.

[4] Albers Rabbit Breeder Ration, Albers Milling Company, Fort Lupton, Colorado.

a period of 20 hours. Two spot specimens were collected by catheterization at 22 and 24 hours and their specific activities were determined. The exchangeable magnesium content of the body was calculated in the manner previously described (Aikawa et al., '59).

Samples of plasma, urine, and tissues were assayed for gamma ray activity with a Nuclear-Chicago model DS-3 well-type scintillation counter which was connected to a Packard Auto-Gamma Sample Changer. All determinations were corrected for physical decay.

The magnesium concentration in urine and serum (Aikawa and Rhoades, '59) and in tissues (Stutzman, '52) was determined by a modification of the molybdivanadate method for phosphate.

The experiment was conducted as follows. During a control period of three days the 8 animals in the test group were fed a balanced stock diet of compressed pellets, containing 172 mEq of Mg per kilogram. Baseline data for body weight, serum Mg concentration, and urinary excretion of Mg were obtained daily. On the 4th day each animal's Mg_e was determined.

After fasting overnight, the animals were then given free access to the Mg-deficient diet for 30 days. The body weight, food consumption, and urinary volume were recorded daily. Mg_e and serum Mg determinations were made on the 8th, 14th, 22nd, and 30th days of the experimental period. On the 31st day, 24 hours after the last intravenous dose of Mg^{28}, the animals were killed by air embolism and the following tissues were assayed for radioactivity and magnesium content: plasma, skin, bone, kidney, appendix, heart, liver, lung, and muscle.

The 4 rabbits in the control group were maintained with the stock diet. They, too, were killed by air embolism 24 hours after the administration of Mg^{28}, and the same tissues were assayed for relative radioactivity and magnesium content.

In the statistical analysis of the external balance data, each animal served as its own control. After the differences between the baseline values and the values obtained during each week had been calculated for each animal, the mean differences for the whole group at each time interval were obtained. The significance of the mean differences was determined by the use of the t test (Snedecor, '46), a P of less than 0.01 being considered significant.

The mean relative activity (see footnote of table 2) and magnesium content of each type of tissue were determined for both the test and control groups, and the significance of the differences between group means was tested (Snedecor, '46).

RESULTS

By the end of the first week that the rabbits received the Mg-deficient diet, body weight had decreased significantly; after the second week the animals began to gain slightly, but did not approach their baseline weights (table 1). With the intake of Mg abruptly decreased to less than 0.3 mEq per day, the renal excretion of Mg fell below 0.8 mEq per day. By the end of the second week the urine volume had

TABLE 1

Effect of magnesium-deficient diet on external balance and exchangeable content of magnesium in 8 rabbits

	Mean baseline values	Mean values at end of			
		Week 1[1]	Week 2[2]	Week 3[3]	Week 4[4]
Weight, kg	1.769	1.596*	1.604*	1.637*	1.622
Serum Mg, mEq/l	2.0	1.2*	1.2*	1.3*	1.2*
Exchangeable Mg content, mEq	58.0	21.0*	13.2*	10.8*	9.5*
Exchangeable Mg content, mEq/kg	33.0	13.2*	8.2*	6.6*	6.0*
Mg intake, mEq/day	21.9	0.17	0.19	0.22	0.13
Urine volume, ml/day	138	105	61*	36*	31*
Urine Mg excretion, mEq/day	10.8	0.79*	0.35*	0.30*	0.54*

[1] Fed Mg-deficient diet for 8 days.
[2] Fed Mg-deficient diet for 14 days.
[3] Fed Mg-deficient diet for 22 days.
[4] Fed Mg-deficient diet for 30 days.
* Statistically significant difference when compared with mean baseline value.

decreased significantly. The serum magnesium concentration dropped to 1.2 mEq per liter by the end of the first week, and remained low. The Mg_e had decreased to one-third of the control value at the end of the first week; it continued to decline progressively, and at 30 days was less than 20% of the control value.

The animal's appearance and behavior was normal until the 4th week, when two rabbits began to lose hair from the back, hind legs, and tail. The coat lost its luster and appeared ragged. At no time did any animal become hyperirritable.

Only in the skin and bone of animals in the test group was there a significant decrease in *relative radioactivity* (table 2). The *magnesium content* was significantly decreased in the lung and bone of rabbits on the Mg-deficient diet (table 3).

TABLE 2

Effect of magnesium-deficient diet on relative radioactivity of tissues in rabbits

Tissue	Relative radioactivity[1]	
	Control group[2]	Test group[3]
Muscle	2.06 ± 0.16[4]	1.42 ± 0.15
Skin	3.28 ± 0.13	1.95 ± 0.24*
Kidney	8.16 ± 0.38	10.61 ± 1.14
Heart	9.46 ± 0.55	12.25 ± 1.29
Liver	10.02 ± 0.62	11.49 ± 1.24
Appendix	12.26 ± 0.67	13.28 ± 1.52
Bone	33.73 ± 2.47	20.70 ± 2.59*

[1] Relative radioactivity = $\frac{\text{count/min./gm tissue}}{\text{count/min./ml plasma}}$ 24 hours after intravenous injection of Mg^{28}.
[2] Mean values in 4 rabbits fed a stock diet.
[3] Mean values in 8 rabbits fed a magnesium-deficient diet for 30 days.
[4] Mean ± standard error.
* Statistically significant difference when compared with control group.

TABLE 3

Effect of magnesium-deficient diet on tissue content of magnesium in rabbits

Tissue	Magnesium content (mEq/kg wet weight)	
	Control group[1]	Test group[2]
Muscle	21.49 ± 0.15[3]	17.90 ± 1.02
Skin	6.35 ± 0.27	5.37 ± 0.32
Kidney	12.23 ± 0.66	11.66 ± 0.39
Heart	12.96 ± 0.40	13.44 ± 0.58
Liver	12.35 ± 0.50	12.88 ± 0.80
Appendix	16.40 ± 0.90	16.74 ± 1.43
Lung	11.75 ± 0.76	8.74 ± 0.45*
Bone	298.10 ± 10.20	251.80 ± 3.89*

[1] Mean values in 4 rabbits fed a stock diet.
[2] Mean values in 8 rabbits fed a magnesium-deficient diet for 30 days.
[3] Mean ± standard error.
* Statistically significant difference when compared with the control group.

DISCUSSION

The results of the external balance study in the present experiment confirm those previously reported by Orent et al. ('34), Watchorn and McCance ('37), Knoop et al. ('39), Duckworth and Godden ('41), Blaxter and Rook ('54), Smith ('59), and Martin and Wilson ('60). Although the urinary excretion of Mg decreased as the intake of this ion was reduced, the net output exceeded the intake. Consequently, the body store of Mg was progressively depleted, and body weight decreased. An abrupt decrease in serum magnesium concentration occurred and persisted throughout the experimental period.

Despite the deficient diet, a significant reduction in tissue Mg content was noted only in bone and lung; because of the relative size of these two organs, it appears likely that the greatest deficiency of Mg must have occurred in bone.

The data on relative radioactivity suggest that the soft tissues continue to take up Mg even when animals are maintained with a Mg-deficient diet for as long as a month; these data confirm those of McAleese et al. ('61) who found that tissues of Mg-deficient lambs had a higher concentration and total amounts of the isotope as compared with control lambs. Our data also suggest that the uptake of Mg is significantly lowered only in the bone and skin. The low Mg content and decreased relative radioactivity in bone confirm the previous impression that the bone serves as the labile store of magnesium in the body. The decrease in the relative radioactivity of the skin may be a factor in producing the edema and hyperemia of the skin associated with Mg deficiency.

That vasodilatation and hyperirritability, characteristic clinical manifestations of magnesium deficiency, did not develop in the animals in the present study may be explained by the diet, which was not as low in Mg as those used in some previous studies, such as that of Orent et al. ('34).

The largest pool of magnesium in the body is in muscle and bone. Since the relative radioactivity and Mg content of muscle were not significantly altered under the conditions of the present experiment, the explanation for the progressive decrease in Mg_e observed in the study lies

primarily in depletion of the labile exchangeable pool of magnesium in bone; a low serum level of Mg may suggest depletion of this labile pool.

SUMMARY

Eight rabbits were fed a magnesium-deficient diet containing 6.6 mEq of Mg per kilogram. As the intake of Mg was decreased, the urinary excretion of Mg dropped to less than 0.8 mEq daily. By the end of the first week the body weight had decreased significantly and the serum magnesium concentration had dropped to 1.2 mEq per liter, where it remained through the 30th day. The exchangeable magnesium content decreased to one-third of the baseline value by the end of the first week, and continued to decrease thereafter. After the animals had received the diet for one month, the magnesium content of most soft tissues was unchanged; only lung and bone showed a significant reduction. At the same time, study of the relative radioactivity content of tissues showed that most soft tissues also continued to accumulate Mg at the usual rate. Relative radioactivity was significantly decreased only in skin and bone. Bone apparently serves as the reservoir from which magnesium is drawn during the ingestion of a Mg-deficient diet.

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GUIDE FOR CONTRIBUTORS

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Weight		*Length*	
kg	kilogram	km	kilometer
gm	gram	m	meter
mg	milligram	cm	centimeter
μg	microgram	mm	millimeter
mμg	millimicrogram	μ	micron
μμg	micromicrogram	mμ	millimicron
		μμ	micromicron
Volume			
m^3	cubic meter		
cm^3	cubic centimeter	*Area*	
mm^3	cubic millimeter	m^2	square meter
l	liter	cm^2	square centimeter
ml	milliliter	mm^2	square millimeter

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A	angstrom units	°	degree
%	per cent	ppm	parts per million

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