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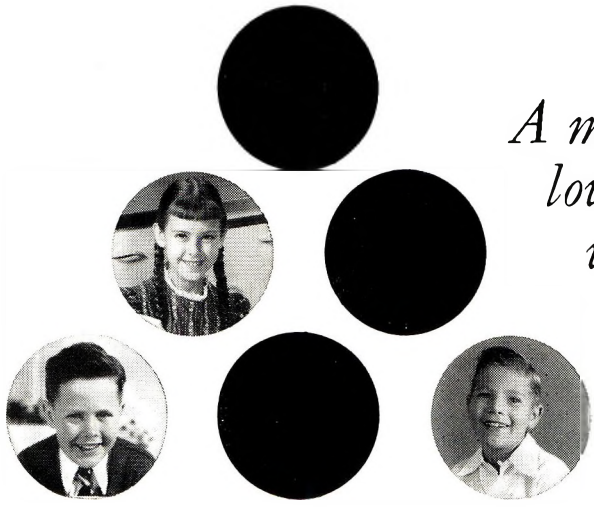

A moderate low-fat well-balanced breakfast for children 10-12 years of age

It has been found that those who develop obesity in childhood appear to have more of a dietary problem than those who develop obesity in the adult years. As a service to those advising about the obese child, a basic cereal and milk breakfast is worth consideration. *Its fat content of 10.9 gm. provides 20 per cent of the total calories, thus it is moderately low in fat.* It is well-balanced as shown in the chart below. The Iowa Breakfast Studies demonstrated that children of about this age have better grades and attitudes at school when they eat a *well-balanced* basic cereal and milk breakfast each morning.

Recommended Daily Dietary Allowances and the Nutritional Contribution of a Basic Cereal and Milk Moderate Low-Fat Breakfast*

Menu: Orange Juice—4 oz.;
Cereal, dry weight—1 oz.;
Whole Milk—4 oz.; Sugar—1 teaspoon;
Toast (white, enriched)—2 slices;
Butter—5 gm. (about 1 teaspoon);
Nonfat Milk—8 oz.

Nutrients	Calories	Protein	Calcium	Iron	Vitamin A	Thiamine	Riboflavin	Niacin equiv.	Ascorbic Acid
Totals supplied by Basic Breakfast	503	20.9 gm.	0.532 gm.	2.7 mg.	588 I.U.	0.46 mg.	0.80 mg.	7.36 mg.	65.5 mg.
Recommended Dietary Allowances—Children, 10 to 12 Years (36 kg.—79 lb.)	2500	70 gm.	1.2 gm.	12 mg.	4500 I.U.	1.3 mg.	1.8 mg.	17 mg.	75 mg.
Percentage Contributed by Basic Breakfast	20.1%	29.8%	44.3%	22.5%	13.1%	35.4%	44.4%	43.3%	87.3%

Cereal Institute, Inc.: Breakfast Source Book. Chicago: Cereal Institute, Inc., 1959.
Food & Nutrition Bd.: Recommended Dietary Allowances, Revised 1958. Natl. Acad. Sci.—Natl. Research Council Publication 589, 1958.
Watt, B. K., and Merrill, A. L.: Composition of Foods—Raw, Processed, Prepared. U.S.D.A. Agriculture Handbook No. 8, 1950.

**The allowance levels are intended to cover individual variations among most normal persons as they live in the United States under usual environmental stresses. Calorie allowances apply to individuals usually engaged in moderate physical activity. For office workers or others in sedentary occupations they are excessive. Adjustments must be made for variations in body size, age, physical activity, and environmental temperature.*

CEREAL INSTITUTE, INC.
135 South La Salle Street, Chicago 3

A research and educational endeavor devoted to the betterment of national nutrition

Studies of the Effects of Dietary NaF on Dairy Cows

IV. DENTAL CHANGES AS THE RESULT OF LONG-TERM FLUORINE INGESTION[1]

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The accumulation of fluorine in osseous tissue is dependent on both the level of dietary fluoride and the duration of exposure (Phillips et al., '55). The completion of two long-term studies of chronic fluorine toxicity in dairy cattle has made available extensive material for studying these variables in dental structures.

The objectives of this study were to investigate the retention of fluorine by the dentin and enamel of incisor teeth in relation to age and eruption, to the length of exposure and to the level of fluorine ingested.

METHODS

Incisors were obtained from cattle which had a known history of controlled ingestion of fluorine. Two conditions of exposure were studied: cows first exposed to supplementary fluorine ingestion at two years of age (809A) and others first exposed at 5 years (809B). In experiment 809A, fluorine was administered as a solution of NaF poured over the grain ration to provide 20, 30, 40, 50 ppm of F, and 50 ppm of F with 200 gm of $CaCO_3$/day. Levels of 30 and 50 ppm of F were used in experiment 809B. The basal diet to which these levels of fluorine were added contained from 3 to 5 ppm of F. Other husbandry practices and results of these studies have been described (Suttie et al., '57a, b, '58; Suttie and Phillips, '59a).

After slaughter the incisors were removed, cleaned and dried at 110°C. The teeth were powdered in a steel mortar until all particles would pass a 60-mesh screen, and enamel and dentin were separated by the method of Manly and Hodge ('39). Fluorine analyses on the dry teeth were carried out by the Alcoa Research Laboratory modification[2] of the Willard and Winter method ('33) and acid solubility determined as described by Walsh et al., ('57), with liberated phosphate measured (Fiske and Subbarow, '25) as the criterion of solubility.

RESULTS

The results of single fluorine analyses of the incisor enamel and dentin are summarized in tables 1 and 2. These data show that graded increments of 20 to 50 ppm of fluorine added to the diet caused an increased storage of fluorine in the incisors. The data indicate that the normal fluorine content of the dentin from these mature cows, all over 7 years old, was from 500 to 1000 ppm and the enamel contained 50 to 300 ppm of fluorine.

The fluorine content of the dentin increased with each increment of fluorine added to the diet and also increased progressively from the first to the 4th pair of incisors. At comparable dietary fluorine intakes there was more fluorine in

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We wish to acknowledge the services of Dr. Thaddeus Kowalczyk D.V.M. for handling the health problems throughout these studies.
[2] Aluminum Company of America, Aluminum Research Laboratories 1947 Technical paper 914.

TABLE 1

Effect of level of dietary fluoride (NaF) upon incisor tooth score and incisor fluorine concentration from cows exposed for 5½ years

Lot	F added	Cow	Dentin of incisor no.				Enamel of incisor no.				Score[1] for incisor no.	
			1	2	3	4	1	2	3	4	3	4
	ppm		*ppm F dry weight*				*ppm F dry weight*					
1	0	2	554	695	991	990	182	226	163	290	1A	1B
		3	498	532	874	850	326	109	163	236	1A	1A
		4	595	777	828	1100	164	—	235	326	1B	1B
2	20	5	1095	1550	1585	2620	174	189	229	416	1B	1B
		6	1575	2060	2860	3320	213	161	662	1210	1B	2
		7	1740	1980	2560	3390	277	319	178	980	1B	4
		8	1285	1545	1870	2080	228	208	865	488	1B	4
3	30	10	1850	1975	2380	4400	426	396	411	1440	1B	5A
		11	2130	2930	3840	4770	434	393	520	1530	3	4
		12	1970	3040	4030	4800	396	272	1070	2350	3	4
4	40	13	2780	3850	5500	6300	495	523	2700	2950	5B	5C
		14	2320	2600	4900	5720	217	226	1920	3000	4	5B
		15	2320	2840	3940	4920	291	554	526	1830	3	5A
		16	2090	2830	4180	—	163	249	1240	2250	4	5A
5	50	17	2510	3520	5970	6570	301	366	290	2780	5B	5C
		19	2420	3240	3670	4520	206	423	595	2840	5B	5C
		20	2250	2520	4270	6270	193	290	167	1140	2	5A
6	50+	22	2160	2320	2900	5180	266	842	248	874	2	4
	200 $CaCO_3$/	23	2400	4320	5060	6230	430	493	1660	2640	5B	5B
	cow/day	24	2510	3690	5660	6370	235	258	1190	2480	4	5B

[1] Scored by the University of Tennessee Agricultural Experiment Station Classification Scheme (Phillips et al., '55).

TABLE 2

Effect of level of dietary fluoride (NaF) upon incisor fluorine concentration of cows exposed for three years (exp. 809B)

Lot	F added	Cow	Dentin of incisor no.				Enamel of incisor no.			
			1	2	3	4	1	2	3	4
	ppm		*ppm F dry weight*				*ppm F dry weight*			
1	0	25	320	371	427	414	102	64	108	145
		26	578	700	1000	810	93	107	164	—
		27	351	398	284	640	127	66	49	99
		28	363	366	332	415	183	61	73	163
2	30	29	1180	1050	1340	1210	255	194	161	178
		30	950	900	1060	2050	135	154	167	185
		31	925	974	1060	1075	126	153	155	188
		32	740	850	917	1260	100	138	169	186
3	50	33	1290	1350	1770	2240	244	203	190	228
		34	915	1580	2930	3820	118	153	306	510
		35	920	1760	2070	2060	184	—	324	480
		36	1110	1200	1360	1870	147	150	193	235

the dentin from the cows exposed at two to 7 years of age (809A) than from cows exposed at 5 to 8 years of age (809B).

The distribution of fluorine in the enamel of different teeth did not follow the same uniform pattern as in the dentin and the variation within each dietary level was much greater. There was no uniform and regular increase in enamel fluorine from the first to the 4th pair of incisors. Rather, the data indicated that in the cows first exposed at two years of age (809A) there was a sharp increase in the fluorine concentration of the enamel in the third or 4th incisor. In experiment 809B, using cows first exposed at 5 years of age, there was little correlation of fluorine intake with either the numerical position of the incisor or level of fluorine intake.

Previous studies (Hobbs et al., '54; Stoddard et al., '54; Newell and Schmidt, '58; Suttie et al., '58; Suttie and Phillips, '59a, b), have demonstrated that the age of the animal as well as the level of fluorine in the diet and duration of exposure will influence the amount of fluorine retained in the bones. In these studies the two groups of cattle were exposed to elevated fluorine ingestion for different periods of time. Therefore, no quantitative measure of the influence of age on fluorine retention in dental tissues would be permissible. However, the data indicate that the younger cows (809A) were able to retain more fluorine in the dentin at comparable levels of intake than the more mature animals (809B). The effect of age was further demonstrated by the variation in the fluorine content of different incisors from the same animal. In experiment 809A, the 4th incisors which were the last to be formed and erupted, retained more fluorine even though in some cases they were exposed for a shorter period of time. This effect on the fluorine retention in the dentin was also evident in the mature cows (809B) in which all the incisors had erupted prior to fluorine exposure.

The data on enamel fluorine concentrations support the observations which indicate that after calcification and eruption the enamel possesses little metabolic activity (Ham, '57). The data in table 2 indicate very little influence of either fluorine intake or numerical position of the incisor on the retention of fluorine in the enamel of the mature cow. In experiment 809A, however, the third and 4th incisors show greatly elevated enamel fluorine concentrations indicating that these incisors were still developing and not yet mature structures when the fluorine was first included in the diet.

The clinical observable changes in the incisors associated with a fluorine toxicosis have received a great deal of attention, and classification schemes have been published (Phillips et al., '55). Fluorine

ingestion can affect only those teeth which are in the formative stage at the time of exposure and therefore only the third and 4th incisors of the cows in the 809A experiment were affected. The other teeth in this experiment as well as all incisors in experiment 809B were normal. The ratings given these affected teeth at the time of slaughter are presented in table 1, and photographs of the teeth from three of the cows receiving 50 ppm of F as well as a control animal are shown in figure 1.

Studies with human dental tissues have indicated a close correlation between decreased acid solubility and fluorine content. Data on the acid solubility of dentin from the cows in experiment 809A are summarized in table 3. These data indicate that increasing the fluorine content of the diet led to a decrease in acid solubility. This effect was most pronounced in the 4th incisors which were exposed to fluorine during their formation and development, thereby accumulating more fluorine.

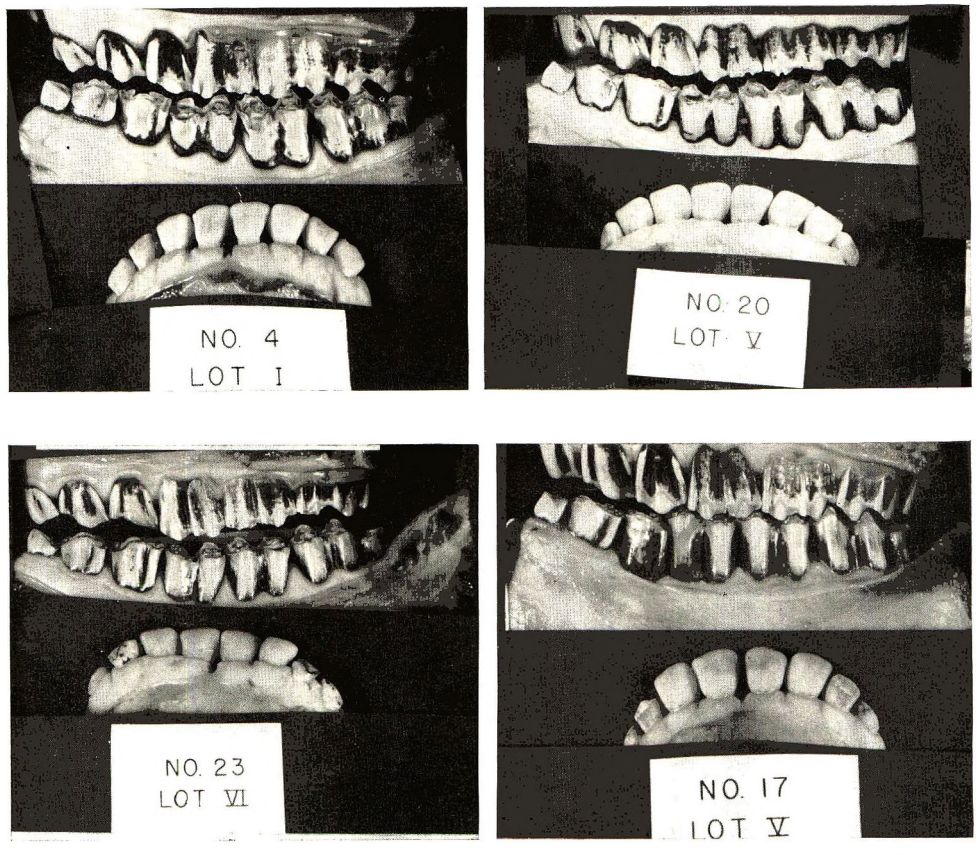

Fig. 1 Cow no. 4. Control low fluorine ration. A normal set of incisor and molar teeth showing wear which is normal for an 8-year old cow. Cow no. 20. Basal ration with 50 ppm of fluorine added as NaF for 5½ years beginning at two years of age. Slight wear on premolars 1 and 2, and on lower molar 3, which is not uncommon in an 8-year old cow. Only the no. 4 incisors were badly affected and the cow was quite resistant to long term fluorine ingestion. Cow no. 23. Same ration as cow 20 with 200 gm of $CaCO_3$ daily. Incisor teeth no. 3 and 4 were badly worn with hypoplastic enamel while the molars were worn more than in either cow 17 or 20. Cow no. 17. A lot mate to cow 20. The third and 4th incisors were badly affected. However, the attrition line was normal for a cow of this age.

TABLE 3

Effect of level of dietary fluoride (NaF) upon acid solubility of incisor dentin from cows exposed for 5½ years (exp. 809A)

Lot	F added	Incisor no.			
		1	2	3	4
	ppm	*mg P/50 mg/30 min*			
1	0	3.57 ± 0.11[1]	3.75 ± 0.06[1]	3.74 ± 0.19[1]	3.72 ± 0.01[1]
2	20	3.38 ± 0.34	3.22 ± 0.18	3.04 ± 0.23	2.71 ± 0.16
3	30	3.41 ± 0.27	2.91 ± 0.15	2.70 ± 0.20	2.52 ± 0.09
4	40	3.19 ± 0.10	2.78 ± 0.10	2.57 ± 0.26	2.37 ± 0.11
5	50	3.06 ± 0.07	2.78 ± 0.26	2.53 ± 0.22	2.22 ± 0.08
6	50+ $CaCO_3$	3.05 ± 0.03	2.82 ± 0.30	2.63 ± 0.35	2.27 ± 0.12

[1] Standard deviation

DISCUSSION

The concentrations of fluorine in the bones of these cattle have been reported previously (Suttie et al., '58; Suttie and Phillips, '59a). In general, the fluorine concentrations found in the dentin were considerably lower than in the bones. However, the study of the younger cows, 809A, showed that the concentration of fluorine in the dentin of the third and 4th incisors approaches that of the metacarpal and metatarsal bones.

The appearance of severely affected teeth is not in itself a reliable criterion upon which to make a diagnosis of fluorine toxicosis. In these experiments dental effects of grade 5A or worse were encountered well before other systemic symptoms of the disease such as: loss of appetite, emaciation, exostoses and stiffness in walking, and concentrations of fluorine in the leg bones in excess of 5500 ppm. This time interval between the dental effects and subsequent systemic effects has been referred to by Peirce ('40) as "the latent period." At the time of slaughter, the molar wear in all animals was normal for cows of their age (fig. 1).

There was no consistent relation between the severity of fluorosis in the incisors and the development of clinical symptoms of fluoride toxicity. At the termination of experiment 809A, cow 17 showed positive symptoms of fluoride toxicosis while cow 19 showed severe symptoms earlier but had recovered at the end of the experiment. Cows 13, 16, 20, 22 and 24 at various times appeared to show signs of marginal toxicosis. There were, however, other cows receiving the same intake of fluoride and having as high or higher fluoride levels in the incisors as the cattle showing signs of toxicity. In spite of this, the latter animals did not develop any systemic symptoms of fluoride toxicosis. This variation of response to a similar intake of fluorine again points out that dental score or any other single symptom cannot be used for a diagnosis of fluorine toxicity.

SUMMARY

The distribution of fluorine in the enamel and dentin of cows exposed to elevated dietary fluorine levels from 2 and 5 years of age through 8 years has been studied. Dentin from control cows of this age contained less than 1000 and enamel less than 500 ppm of fluorine.

The level of fluorine in the dentin, much like bone, increased with added increments of dietary fluorine and with duration of exposure. The amount of fluorine in the dentin increased from the first to the 4th incisor, indicating a more rapid accumulation in younger osseous structures.

Increasing the dietary level of fluorine resulted in increased enamel fluorine only if the teeth had not erupted prior to exposure.

Dentin having an increased fluorine concentration was shown to also have a decreased solubility in acid.

Incisors which were in the formative stage during the fluoridation period developed typical signs of dental fluorosis. However, dental fluorosis, which usually

precedes other systemic symptoms of fluorine toxicity, is quite variable and should not in itself be used as a definitive index of fluorine toxicosis.

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Chick Tissue-Storage Bioassay of Alpha-Tocopherol: Chemical Analytical Techniques, and Relative Biopotencies of Natural and Synthetic Alpha-Tocopherol[1]

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The prevention of fetal resorption in the rat (Mason, '42) has been the most generally used bioassay for vitamin E. By this type of bioassay, Harris et al. ('44), contrary to the belief at that time, found that the natural tocopherols had a greater biological potency than the synthetic. Later, Harris and Ludwig ('49a), who conducted rat pregnancy studies, reported that natural *d*,α-tocopherol was 1.36 times more potent than the synthetic *dl*,α-tocopherol. Using a different criterion of measurement in the rat, the dialuric acid hemolysis method, Friedman et al. ('58) reported that the natural form was 1.33 times more potent than the synthetic. Hove and Harris ('47), by feeding tocopherol to cure muscular dystrophy in rabbits, observed that the natural α-tocopherol was 1.22 times more effective than the synthetic.

Agreement concerning the relative potencies between the free and acetate esters of the natural and synthetic α-tocopherols, however, is not good. Rat pregnancy studies by Harris and Ludwig ('49b) showed that the esters of *d*, and *dl*,α-tocopherol were 1.62 times more effective than the respective free tocopherols. The dialuric acid hemolysis method, as used by Friedman et al. ('58), showed equal utilization between the free and esterified *dl* forms of α-tocopherol. Week et al. ('52), using oral doses of synthetic tocopherols in humans and measuring the increase in plasma tocopherol, reported that the *free* form was 35% more effective than the ester.

Thus, bioassay methods for the tocopherols have been used for three species of animals, with 4 criteria of measurement. Based upon the extensive work of Harris and Ludwig, the 10th revision of the National Formulary stated that *d*,α-tocopherol or its acetate ester is 1.36 times more potent in anti-sterility effect on rats than *dl*,α-tocopherol or its acetate ester.

By using improved chemical methods for the determination of tocopherol in animal tissue, Bunnell ('57) showed that the tocopherol stores in the liver of the chick varied linearly with the intake of α-tocopherol.

The growing importance of vitamin E in nutrition has prompted the workers in this laboratory to use a chick bioassay to evaluate the relative potencies of the *d* and *dl* forms of α-tocopherol and their acetate esters.

EXPERIMENTAL

Day-old White Plymouth Rock male chicks were placed in electrically heated, wire batteries and fed a tocopherol-low diet (Singsen et al., '54) for 13 days. This tocopherol-low diet is presented in table 1. The smallest and largest chicks, approximately 40% of the total, were then removed, and the remaining chicks were distributed into experimental groups of 8 chicks of approximately equal weight. The basal diet, supplemented with 8 and 16 mg of the appropriate tocopherol per pound of diet, was fed on a restricted basis for

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TABLE 1

Composition of the vitamin E-low basal diet

Ingredient	Amount
White corn meal[1]	68.32
Soybean oil meal (50% protein)	10.00
Crude casein	15.00
Fish meal (60% protein)	2.50
Dicalcium phosphate	2.00
Ground limestone	1.50
Sodium chloride	0.50
Manganese sulfate	0.0125
Vitamin supplement in grams per 100 pounds of diet	
Vitamin A[2] (325,000 I.U./gm)	1.7461
Vitamin D_3 (3,000 I.C.U./gm)	22.7
Choline chloride	40.0
Biotin	0.0045
Niacin	1.500
Riboflavin	0.227
Ca pantothenate	0.635
Pyridoxine·HCl	0.227
p-Aminobenzoic acid	0.227
Inositol	0.227
Thiamine·HCl	0.090
Folic acid (3%)	0.605
Vitamin B_{12} (1 mg/gm)	0.600
Vitamin K (menadione)	0.045

[1] A de-germed, de-branned product manufactured by the Quaker Oats Co., Chicago.

[2] Dry vitamin A acetate beadlets manufactured by Hoffmann-LaRoche, Inc., Nutley, New Jersey.

14 days. The same tocopherol samples were used in all replications of the experiment. The natural (*d*,α-tocopherol, *d*,α-tocopheryl acetate[2]) and synthetic (*dl*,α-tocopherol, *dl*,α-tocopheryl acetate) tocopherols were used in the form of fine gelatin sugar beadlets and added directly to their respective diets on an equimolar basis equivalent to tocopheryl acetate.[3] Feed was restricted severely for the first day, approximately 7 gm per chick, then regulated to the amount of diet consumed by the group of chicks with the least appetite. Feed consumption on the last day of the experimental period was approximately 38 gm per chick. At the end of the supplementation period, blood samples were taken by heart puncture; 3 ml were removed from each chick and all 8 samples from each group pooled. The birds were then decapitated to insure more complete elimination of blood from the liver. The pooled livers from each group were removed, homogenized in a high speed blender, sampled into extraction thimbles and frozen at −20°C in tightly stoppered plastic tubes.

METHODS

Since the method used for tissue tocopherol analysis in this laboratory consists of parts of a number of methods which are scattered throughout the literature, it was felt desirable to compile these various processes, along with some modifications in technique, and incorporate them into one publication. Using this method, recoveries of 95% or better were obtained when tocopherol standards were added to liver samples.

Reagents

Purified Skellysolve B. Approximately 1500 ml of crude Skellysolve B were shaken vigorously in a two-liter separatory funnel with three to 4 100-ml portions of concentrated sulfuric acid, or until the acid was colorless after shaking. The acid was drawn off, the Skellysolve was washed once with water, then shaken vigorously with 100 ml of saturated potassium permanganate to which 1 to 2 ml of concentrated sulfuric acid had been added. (CONCENTRATED SULFURIC ACID SHOULD NOT BE ALLOWED TO LAYER OVER OR STAND IN CONTACT WITH POTASSIUM PERMANGANATE CRYSTALS.) All the permanganate was washed out with water, and the solvent was dried over Drierite for 24 hours. It was then distilled, the first and last 10% of the portions being discarded.

Absolute ethanol. Ethanol was distilled over potassium hydroxide pellets and potassium permanganate in an all glass apparatus, and the first and last 20% of the portions were discarded.

2,2′ Bipyridine solution. One-half (0.50) gm of 2,2′ bipyridine was weighed into a glass-stoppered 100-ml low-actinic volumetric flask and made up to volume with purified absolute ethanol. This solution

[2] The natural form after being esterified for increased stability.

[3] The authors are indebted to the Floridin Company, Tallahassee, Florida, for the Florex XXS; to Distillation Products Industries, Rochester, New York, for the natural tocopherols; to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for the synthetic tocopherols and for processing the various tocopherols into beadlets.

was stored in a refrigerator but brought to room temperature prior to use.

Ferric chloride solution. Two-tenths (0.20) gm of $FeCl_3 \cdot 6H_2O$, reagent grade, was weighed into a 100-ml glass-stoppered low-actinic volumetric flask and made up to volume with purified absolute ethanol. This solution was stored in a refrigerator but brought to room temperature prior to use.

Potassium hydroxide solution. Forty gm of potassium hydroxide, reagent grade, were dissolved in 27 ml of distilled water.

Benzene. Baker's analyzed.

Sodium sulfate. Anhydrous, reagent, granular.

Stannous chloride. Reagent grade.

Florex XXS. 60 to 90 mesh.

Extraction

The method of extraction used was basically that described by Quaife et al. ('48, '49). An extraction thimble, containing the sample, was placed into a siphon extraction cup which was suspended by means of nichrome wire from the inner hooks of a Standard Taper 55/50 inner joint of a reflux condenser. A boiling chip and 75 ml of absolute ethanol were placed into a 500-ml Erlenmeyer flask equipped with a Standard Taper 55/50 outer joint. The flask was placed on a multiple unit extraction hot plate at high heat. After extracting for 20 hours in complete darkness or subdued light, the flask was cooled rapidly to room temperature, and approximately 2 gm of anhydrous sodium sulfate, 65 to 70 ml of water, and exactly 40 ml of Skellysolve B were added. The flask was stoppered with a Standard Taper 55/50 inner stopper (the joint was first wetted with water) and shaken in a mechanical shaker for 20 minutes. When the layers separated after a few minutes of standing, the aqueous layer was aspirated off and the remaining Skellysolve extract was quickly transferred to a glass stoppered centrifuge tube and centrifuged for 5 minutes. The above and subsequent operations were performed under subdued light.

Molecular distillation

A description of the pot still and its critical dimensions were reported by Quaife et al. ('46, '48). An aliquot of the Skellysolve B extract from the extraction procedure was pipetted into an aluminum sample-holder and evaporated with the aid of heat and a stream of nitrogen. The sample was placed into the pot of the still and degassed at a pressure of 10 μ of mercury or less for 5 minutes. The vacuum was released and the condenser and aluminum liner, which contained ethanol or acetone, were inserted. After the vacuum pump was operating, dry ice was added to the solvent in the aluminum liner. The oil bath, which contained Dow Corning 550 silicone oil, was raised so that the surface of the oil was even with or slightly above the bottom of the condenser, and a pressure of 10 μ or less was maintained. The distillation was allowed to proceed for 30 minutes at a temperature between 220 to 225°C. At the end of the distillation period, the oil bath was lowered. While the glass pot was cooling, 25 ml of benzene were pipetted into a 100-ml beaker and covered with a watch glass. The aluminum liner, which contained the dry ice and solvent, was removed from the condenser, and warm water (about 50°C) was added to prevent the condensation of moisture on the cold distillate when the vacuum was released. The vacuum was then released and the distillate removed completely by carefully swirling the condenser in the benzene. This benzene solution was poured into a 50-ml Erlenmeyer flask and stoppered. An aliquot of this solution was taken for the subsequent chromatographic procedure. Extreme care was exercised to prevent the solvent from coming in contact with rubber or cork, since reducing substances would be extracted which would interfere with the analysis.

Chromatography

The chromatographic method used was essentially the procedure described by Brown ('52) and Swick and Baumann ('52). Sixteen gm of Florex XXS and 2.0 gm of $SnCl_2$ were weighed into a 250-ml Erlenmeyer flask, then 60 ml of 10 N

HCl were added. This was sufficient Florex to make 4 columns. This was boiled for approximately one minute. The hot solution was poured into the 4 chromatography tubes (body of tube—14 mm inside diameter, 125 mm long) into which plugs of glass wool had been previously placed. The adsorbent and the sides of each tube were washed down, first with 5 ml and then 3 ml of absolute ethanol. This was followed with 5 washes of 5 ml of benzene. After the third addition of the benzene, the adsorbent was stirred up with a thin stirring rod and then packed down firmly with the flattened end of a glass rod. After the last portion of benzene had passed through, an aliquot of the sample from the molecular distillation procedure was introduced and eluted with 5-ml portions of benzene until a 25-ml volumetric flask was brought to volume. A 5- or 10-ml aliquot of the sample was added directly; however, if more than this amount of aliquot was used, it was placed into a 50-ml Erlenmeyer flask and evaporated to a volume of approximately 5 ml by the use of heat and a stream of nitrogen prior to transfer to the Florex column. The Erlenmeyer flask was then rinsed with 5-ml portions of benzene which were transferred to the chromatographic column.

Color formation

An aliquot of benzene from the chromatographic step was placed into a 50-ml Erlenmeyer flask and evaporated to dryness with the aid of heat from a water bath and a stream of nitrogen. The last milliliter was evaporated without heat. The residue was dissolved completely with exactly 10 ml of absolute ethanol and exactly 8 ml were transferred to an Evelyn cuvette. One milliliter of the bipyridine solution was added by use of a serological pipette and mixed by swirling. The Evelyn photoelectric colorimeter was set to read 100% transmission. With another serological pipette, 1 ml of the $FeCl_3$ solution was added, quickly swirled until well mixed, and the percentage transmission read, at 515 or 520 mμ, 15 seconds after addition of the $FeCl_3$ solution. A standard curve was made by preparing standard solutions of *d* or *dl*,α-tocopherol in absolute ethanol, ranging between 5 and 75 μg of tocopherol per 8 ml of solution. The density of a blank, read 15 seconds after addition of the $FeCl_3$ solution, was subtracted from the density of each solution that contained tocopherol. A blank was required each time tocopherol determinations were made. The 15-second color development was considered sufficient by Quaife and Harris ('44) for α-tocopherol to react completely.

Plasma tocopherol

The method used to determine plasma tocopherol was described by Dicks et al. ('57), using the hydrogenation procedure.

Beadlets

Beadlets were dissolved in acidified warm water and extracted three times in a separatory funnel with petroleum ether. The combined extracts were dried by passing through anhydrous sodium sulfate into a round bottom boiling flask and evaporated to dryness with the aid of nitrogen and heat. The acid hydrolysis and ceric sulfate titration procedure was then followed as reported by Lehman ('57).

Feed

Basal diets and diets containing nonesterified tocopherols were analyzed by the same method as described for tissue tocopherol. Diets that contained esterified tocopherol were analyzed as above, except that 4 ml of the potassium hydroxide solution were added to the extraction flask one-half hour prior to the end of the extraction period. At the end of the one-half hour saponification period, the saponification mixture was acidified by adding 65 ml of water containing 2 ml of concentrated sulfuric acid. Sodium sulfate was not added in this procedure since the solution contained a sufficient amount from the neutralization reaction. The analysis was then continued, beginning with the addition of exactly 40 ml of Skellysolve B, as described under the section entitled *extraction.*

RESULTS

The total tocopherol content of the basal diets varied between 4.6 and 7.1 mg per

TABLE 2

Tocopherol values and liver weights obtained by feeding equimolar amounts of natural and synthetic, free and esterified α-tocopherol

Experiment[1]	Equimolar-equivalent to milligrams of tocopheryl acetate per pound of diet								
	dl, α-tocopheryl acetate			*dl*, α-tocopherol		*d*, α-tocopheryl acetate		*d*, α-tocopherol	
	0	8	16	8	16	8	16	8	16
				Liver tocopherol (*μg/gm*)					
119[2]	0.76[3]	4.79	8.40	4.47	8.65	6.38	12.26	—	—
119B	1.73	6.14	10.25	4.76	10.32	7.55	12.64	—	—
119C	1.75	5.68	9.03	5.10	9.84	5.76	12.07	—	—
119D[4]	1.90	5.26	10.63	6.21	10.50	7.60	13.26	6.84	13.38
119E	1.96	6.33	10.38	6.52	9.52	7.80	12.50	7.14	12.25
				Plasma tocopherol (*μg/100 ml*)					
119B	80	295	650	291	548	479	768	—	—
119C	73	295	547	313	593	430	815	—	—
119D[4]	59	264	515	323	459	426	673	405	756
119E	79	394	718	350	680	521	813	481	887
				Liver weight (*gm/8 chicks*)					
119[1]	—	57.6	59.3	61.3	57.4	57.7	60.8	—	—
119B	54.9	51.9	57.6	60.5	56.3	57.6	58.6	—	—
119C	54.6	55.9	56.8	55.1	56.6	55.6	65.9	—	—
119D[4]	77.0	82.6	78.7	81.8	78.8	78.4	82.4	76.1	76.1
119E	74.6	73.4	74.5	75.6	74.7	75.6	76.3	75.3	76.3

[1] Feed consumption per 8 chicks from 13 to 27 days of age in experiments 119 through 119E was 2512, 2880, 2630, 3158 and 3120 gm, respectively.

[2] Average of three replicates.

[3] Calculated missing value.

[4] Average of two replicates.

pound of diet expressed as tocopheryl acetate. Feeding this basal diet for 13 days lowered the tocopherol content of the liver tissue from approximately 150 μg to 3 μg per gm of tissue.

The data presented in table 2 represent 5 experiments in which liver was analyzed for tocopherol and 4 experiments in which plasma was analyzed for tocopherol. A direct relationship can be observed between the dietary tocopherol level and the response as measured by the tocopherol content of the liver. The relationship was found to be linear, in agreement with a similar observation reported by Bunnell ('57). Slopes of these lines were compared with the slope produced by *dl*,α-tocopheryl acetate and expressed as ratios. These data, both for liver and plasma tocopherol, were analyzed by the multiple slope ratio assay as described by Finney ('52). The model is essentially $Y = a + b_s x_s + bu_1 xu_1 + bu_2 xu_2 + \ldots bu_n xu_n$. In solving the equations, the abbreviated Doolittle method, as given in Anderson and Bancroft ('52), was used. By solving the equations for the 5 experiments in which liver tocopherol was the response measured, the following slope ratio was obtained:

$$\frac{d,\alpha\text{-tocopheryl acetate}}{dl,\alpha\text{-tocopheryl acetate}} = 1.34$$

with 95% confidence limits between 1.26 and 1.42. The data for plasma tocopherol, analyzed by the multiple slope ratio assay, resulted in a ratio of 1.36 with 95% confidence limits between 1.23 and 1.50.

When the free *dl*,α-tocopherol was compared with its ester, using liver tocopherol as the criterion of measurement, the following ratio was obtained:

$$\frac{dl,\alpha\text{-tocopherol}}{dl,\alpha\text{-tocopheryl acetate}} = 0.99$$

with 95% confidence limits between 0.92 and 1.06. The same comparison, but measuring the tocopherol content of plasma, gave a ratio of 0.95 with 95% confidence limits between 0.84 and 1.06. The ratio comparing *d*,α-tocopherol with its acetate ester was not determined by the multiple slope ratio assay because the beadlets containing *d*,α-tocopherol were not available until the last two experiments were run. The averages of the slope ratios in the last two experiments, when liver and plasma were used as the criteria of measurement, were 0.97 and 1.01, respectively. Confidence limits were not determined because of insufficient replication.

DISCUSSION

From the data, it would appear that the chick utilized *d*,α-tocopheryl acetate equally as well as the rat when the comparison was made with *dl*,α-tocopheryl acetate. Although plasma tocopherol could be used for measuring the relationship between the *d* and *dl* forms, a greater variation was associated with it than there was with the liver tocopherol. The acetate ester, on the other hand, was utilized equally as well as the free form; again, a greater variability was noted with plasma tocopherol. This relationship occurred whether the natural or the synthetic tocopherol was being compared with its respective acetate ester. In rat pregnancy assays, the ratio of *d*,α-tocopheryl acetate to *d*,α-tocopherol was reported to be 1.62. It was interesting to note that Friedman et al. ('58), using the same species but a different criterion of measurement, obtained equal utilization.

Although the chick bioassay, as with most bioassay methods, is not a short method, it does have an advantage in that it does not involve keeping a breeding stock of animals. Also, no specialized selection is required, for in these experiments one breed of chicks was obtained from three hatcheries. The experiments were carried out over all seasons with essentially good replication, and final values, favorably comparable with rat bioassays, were obtained.

It is believed that with some modification in experimental procedure and technique, the time required to obtain a final answer could be reduced and precision in analytical determinations could be further improved.

SUMMARY

A chick bioassay method is described for comparing the potencies of *d* and *dl*, α-tocopherol with their respective acetate esters. It was found that *d*,α-tocopheryl acetate was 1.34 times more potent than

dl,α-tocopheryl acetate as measured by liver tocopherol content, and *d* or *dl*,α-tocopherol were utilized equally as well as their respective acetate esters.

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The Absence of Hemorrhagenic Compounds in Irradiated Beef[1]

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When irradiation-sterilized beef was fed to weanling rats, a large number of male animals died from severe hemorrhages. Metta and associates ('59) found that such hemorrhages could be prevented by oral administration of menadione. It appears possible that irradiation-induced chemical reactions could change the structure of natural quinones or other compounds in beef, transforming them into vitamin K antagonists. Nevertheless, severe vitamin K deficiency has been produced, as well as hemorrhages, in rats fed a purified diet containing no antagonist (Mameesh and Johnson, '59a). Furthermore, in contrast with the human species, the vitamin K requirement of the rat can be satisfied by means of coprophagy (Barnes et al., '59; Mameesh and Johnson, '59). It thus seemed important to study the mechanism by which vitamin K deficiency occurred in rats fed irradiated beef, since only when this is understood can the safety of feeding irradiated beef to humans be evaluated.

MATERIALS AND METHODS

The possible occurrence of vitamin K antagonist(s) in irradiated[2] ground beef was investigated by two methods: (1) examination of the hemorrhagic effect of feeding the irradiated beef at several levels in the diet, and (2) determination of the vitamin K requirement of rats fed irradiated beef in the diet, comparing it with the requirement of those fed a purified diet. Ground beef irradiated at 2.79 and 5.58 million rad was supplied to us in sealed cans by the Department of the Army. The beef was cooked in an autoclave for 25 minutes at 17 pounds pressure, and then

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[2] Irradiation with gamma rays was carried out at the Atomic Energy Commissions' Material Testing Reactor (MTR) at Arco, Idaho, by the Phillips Petroleum Company.

TABLE 1

Effect of increasing levels of irradiated beef; composition of diets

Constituents	Diet A	Diet B	Diet C
	%	%	%
Irradiated beef (2.79 million rad[1])	42.0[2]	21.0[2]	10.5[2]
Soy protein[3]	—	17.0	25.5
Refined corn oil[4]	—	4.0	6.0
Sucrose	18.0	18.0	18.0
Starch	29.0	29.0	29.0
Cod-liver oil	1.5	1.5	1.5
Wheat germ oil	0.5	0.5	0.5
Salt mixture no. 446[5]	4.0	4.0	4.0
Glucose, vitaminized[6,7]	5.0	5.0	5.0

[1] Rad is defined as 100 ergs of radiant energy absorbed per gram of the substance.
[2] Dry substance; however, wet beef (41% dry matter) was used in the diet.
[3] Drackett Assay Protein.
[4] Mazola, Corn Products Refining Company.
[5] Spector ('48).
[6] Metta et al. ('59).
[7] Vitaminized Cerelose.

TABLE 2

Effect on hemorrhagic syndrome of increasing levels of irradiated beef; mortality data

Diet	No. of rats	Hemorrhagic deaths			
		Week 1	Week 2	Week 3	Total
A[1]	8	0	0	2	2
B	8	0	0	6	6
C	8	1	3	2	6

[1] See table 1.

incorporated wet into three diets as described in table 1. The diets contained, by calculation, approximately the same protein and fat content on a dry matter basis. Soy protein[3] and purified corn oil were used to equalize the diets in protein and fat.

Weanling male albino rats of the Sprague-Dawley strain were housed in individual wire-bottom cages in a temperature-controlled laboratory and fed the experimental diets ad libitum for three weeks. The rats were examined daily for hemorrhages, and hemorrhagic deaths were confirmed by autopsy and recorded. The results of this experiment are given in table 2.

The vitamin K requirement for the maintenance of normal blood coagulation of rats fed diets containing irradiated beef (5.58 million rad) was determined using the diet shown in table 3. Vitmain K_1 (3-phytyl menadione) was either mixed with the diet or administered orally as a solution in glyceryl trioleate by a calibrated eye-dropper. In either case, the vitamin was given at the levels of zero, 0.05, 0.10, 0.20 and 0.40 μg per gm of diet on a dry matter basis. The experimental diets and the vitamin solutions were prepared every two weeks, kept under refrigeration and protected from light. In order to rule out any effect of coprophagy, all rats were fitted with tail cups according to the method of Barnes et al. ('57). The experiment lasted 4 weeks, at the end of which the rats were sacrificed and the plasma prothrombin times determined by the method of

TABLE 3

Basal diet

	gm/100 gm diet
Irradiated ground beef[1] (5.58 million rad)	35.0
Sucrose	35.0
Starch	19.0
Glyceryl trioleate[2]	2.0
Salt mixture no. 446[3]	4.0
Glucose, vitaminized[4]	5.0

[1] Dry substance; however, wet beef was used in the diet.

[2] Contained 0.25 gm methyl linoleate and 12 mg α-tocopherol acetate.

[3] Spector ('48).

[4] Vitaminized Cerelose. (Metta et al., '59); also supplied vitamin A, 2000 U.S.P. units and vitamin D_3, 200 I.U. per 100 gm diet. Obtained from Nopco Chemical Co., Harrison, New Jersey.

[3] Drackett C-1 Protein, Archer-Daniels-Midland Company, Cincinnati.

TABLE 4

Hemorrhagic deaths and plasma prothrombin times of rats fed an irradiated beef diet supplemented with several levels of vitamin K_1

Diet	Oral supplement	No. of rats	Plasma prothrombin time	Hemorrhagic deaths
	μg K_1/gm diet		*mean (spread), seconds*	
6 Million rad beef diet	—	10	32.0 (14–96)	2
6 Million rad beef diet + 0.05 μg K_1/gm diet	—	5	19.4 (14–28)	0
6 Million rad beef diet + 0.10 μg K_1/gm diet	—	5	14.6 (13–17)	0
6 Million rad beef diet + 0.20 μg K_1/gm diet	—	5	14.2 (13–16)	0
6 Million rad beef diet + 0.40 μg K_1/gm diet	—	5	14.2 (13–16)	0
6 Million rad beef diet	0.05	5	19.2 (17–21)	0
6 Million rad beef diet	0.10	5	16.2 (15–18)	0
6 Million rad beef diet	0.20	5	15.4 (14–19)	0
6 Million rad beef diet	0.40	5	14.4 (13–15)	0

Quick ('38). The observations from this experiment are shown in table 4.

RESULTS AND DISCUSSION

When a hemorrhagic syndrome, preventable by vitamin K administration, was encountered in rats fed diets containing irradiated beef and unsupplemented with vitamin K (Metta et al., '59), the presence of hypoprothrombinemic factors in the irradiated beef was suspected on the basis of the prevalent opinion that intestinal microbial synthesis supplies the rat with adequate vitamin K (Owen, '54). However, the recent reports by Barnes et al. ('59) and by Mameesh and Johnson ('59) indicate a dietary vitamin K requirement for rats fed certain purified diets and show that coprophagy is a source of this vitamin.

Table 1 gives the composition of three diets that differed with respect to their content of irradiated ground beef. When these diets were fed to three groups of rats for a period of three weeks, it was found (table 2) that the diets which contained less irradiated beef caused more and earlier hemorrhages. These observations show that irradiated beef did not intrinsically contain hemorrhage-inducing compounds, since if it did, the severity of the hemorrhagic syndrome would increase as the irradiated beef content of the diet is increased. The results indicate also that the soy protein-corn oil mixture, used to equalize the protein and fat content of the diets, was a poorer source of vitamin K than irradiated beef.

In another series of experiments the vitamin K requirement of non-coprophagic male rats fed a diet high in irradiated beef (table 3) was determined. The results show that 0.1 μg of vitamin K_1, per gm of the diet (on a dry matter basis) was adequate to maintain normal plasma prothrombin levels, whereas lower levels of the vitamin were inadequate (table 4). This value for the requirement is the same as that reported previously for non-coprophagic weanling male rats fed a purified diet (Mameesh and Johnson, '60). The results of both these experiments indicate that irradiation of the beef did not produce hemorrhagenic compounds or vitamin K antagonists. Thus it appears that the hemorrhagic syndrome was the result of a lack of vitamin K by rats fed irradiated beef as compared with those fed non-irradiated beef.

It is of interest that the vitamin K requirement was the same whether the vitamin was mixed with the diet or given separately. This indicates that vitamin K_1 is stable for at least two weeks when mixed with a diet containing irradiated beef.

SUMMARY

Studies of the nature of the hemorrhagic syndrome observed in weanling male rats fed irradiated beef indicated that the syndrome was caused by a lack of vitamin K and not by any hypoprothrombinemic factor in irradiated beef. This conclusion was based on the observations that: (1) when the level of irradiated beef in the diet was increased, the incidence and severity of hemorrhages decreased; and (2) the vitamin K requirement of coprophagy-prevented rats fed a diet high in irradiated beef was found to be the same as that reported for similar rats fed a purified diet.

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The Effect of Exercise on Nutrient Utilization with Special Reference to Calcium[1]

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It is generally accepted that the uncertainty associated with optimal calcium requirements exists primarily because the body is able to adapt itself to various levels of dietary intake. The mechanism of this adaptive process is still obscure and much remains to be explored and elucidated although it appears to assert itself, in part, by an alteration in the degree of calcium utilization (Gershoff et al., '58).

The many factors known to influence calcium utilization are discussed in the excellent reviews and papers dealing with calcium metabolism (Nicolaysen et al., '53; Malm, '58). A possible factor which has not received adequate consideration, however, is the influence of exercise. The deleterious loss of calcium from lack of exercise has been known and clearly demonstrated in patients (Howard et al., '45) and in healthy subjects (Deitrick, '48). Merely increasing blood circulation with an oscillating bed reduced the calcium loss by 50% (Deitrick, '48; Whedon et al., '49). The value of exercise in immobilized patients or subjects thus is established. The beneficial effects of additional exercise upon calcium retention also have been reported by Pinkos[3] in studies with young growing dogs, and by Liu and McCay ('53) and Wanner[4] in studies with old-aged dogs.

In the present study, the effect of exercise on calcium utilization in young adult dogs was investigated further by conducting balance studies. The exercise was imposed subsequent to consuming diets relatively high in either protein, fat, or carbohydrate. The utilizations of phosphorus, nitrogen, dry matter, ether extract and carbohydrate also were investigated.

EXPERIMENTAL

Six adult male Beagle dogs, averaging 9.0 kg in body weight and 22 months of age were used in this study. The animals were housed and fed in individual metabolism cages throughout the study. Since the dogs had been accustomed to a commercial diet-mix relatively high in calcium, all animals initially were fed a diet containing only 0.17% of calcium for 25 days prior to the first balance period. This adjustment period also afforded an opportunity to train and familiarize the dogs with the treadmill on which they were exercised. The composition of the three low-calcium diets subsequently fed were identical to those described earlier (Konishi and McCay, '60), distilled water was allowed ad libitum. Experimental procedures, collection of excreta and analysis of samples were as described in the previous report. Apparent absorptions were calculated on the basis of intake minus fecal excretion and retentions were calculated as a percentage of the apparent absorption.

The exercise consisted of a 30-minute run on a motor-driven treadmill set at a gradient of 15.3° and speed of 6.0 km/hour. The activity regimes consisted of a control period without exercise; zero-time, where exercise was begun immediately postprandial; and 60-time, where exercise was begun one hour postprandially. Each dog was exposed to the three activity regimes, the diet being one relatively high in either protein, fat or carbohydrate. The dogs were randomly assigned to a particu-

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[2] Data taken from a part of a Ph.D. thesis submitted to the Faculty of the Graduate School of Cornell University. Present address: U.S. Naval Radiological Defense Lab., San Francisco.
[3] Pinkos, J. A. 1952 Calcium and phosphorus metabolism studies on Great Dane dogs. Ph.D. thesis, Cornell University, Ithaca, New York.
[4] Wanner, R. L. 1955 Calcium metabolism in dogs, with respect to age and exercise. Ph.D. thesis, Cornell University, Ithaca, New York.

lar diet, control and activity schedule according to a $6 \times 3 \times 3$ factorial design.

RESULTS

Observation of the dogs during the exercise periods did not reveal any demonstrable ill-effects; vomiting never occurred during or subsequent to the exercise. Body weights remained essentially the same throughout the study.

The mean intakes, apparent absorptions, excretions and retentions of calcium are summarized in table 1. There was a significantly lower ($P < 0.01$) apparent absorption of calcium during the exercise periods. The phosphorus and nitrogen utilizations were unaffected by this level of exercise. The time of exercise after a meal did not influence the utilization of the nutrients. Urinary excretions of calcium, as well as phosphorus and nitrogen, were not significantly changed during the exercise. The mean volumes of urine excreted during the control, immediate exercise, and one-hour postprandial exercise periods were 538, 529 and 618 ml per day, respectively. Although the volume excreted during the latter exercise period was statistically greater ($P < 0.05$) than during the other two periods, the differences are probably not of physiological significance.

The retention of calcium, phosphorus and nitrogen also were unaffected by the activity. It was interesting to note that although the diet contained as little as 0.17% of calcium, negative calcium balances were never observed.

Contrary to expectations the absorption of calcium was not significantly modified using the high fat diet; although the mean values were lower, the differences between diets did not quite reach the 5% level of statistical significance. The urinary calcium excretions, however, were significantly different ($P < 0.01$) between diets, being highest with the high protein diet and lowest with the fat diet.

The apparent absorbabilities of dry matter, ether extract and carbohydrate were not influenced by the level of exercise imposed.

DISCUSSION

It is said that inhabitants of tropical or semitropical regions customarily take a "siesta" after meals in keeping with the common lay belief that exercise immediately after eating interferes with food absorption. From a nutritional standpoint this practice has been advocated by some for centuries largely on the basis of experience rather than on experiment. Indeed, the majority of the previous studies have evaluated the influence of exercise upon absorption indirectly by observing its effect upon either gastric secretion, acidity, or motility and not upon utilization of nutrients *per se*. In this study, the only nutrient affected by the level of exercise applied was calcium. The decrease in

TABLE 1

Mean intake, apparent absorption, urinary excretion, and retention of calcium of 6 dogs during control and exercise periods using three diets

Diet	Activity period[1]	Intake	Apparent absorption[2]		Urinary excretion		Retention	
		gm/day	%	*S.E.*[3]	*gm/day*	*S.E.*	%	*S.E.*
High protein	control	0.473	60	± 3.5	0.178	± 0.015	37	± 4.4
High protein	0-time	0.472	56	± 3.8	0.163	± 0.012	37	± 5.4
High protein	60-time	0.474	52	± 4.7	0.172	± 0.019	29	± 7.3
High fat	control	0.370	51	± 3.1	0.104	± 0.014	46	± 4.1
High fat	0-time	0.390	46	± 5.1	0.101	± 0.012	42	± 5.2
High fat	60-time	0.381	46	± 4.2	0.092	± 0.011	45	± 8.0
High carbohydrate	control	0.407	55	± 5.1	0.126	± 0.018	44	± 5.0
High carbohydrate	0-time	0.396	53	± 3.0	0.121	± 0.018	43	± 5.5
High carbohydrate	60-time	0.396	50	± 5.4	0.128	± 0.010	33	± 6.2

[1] Control, no exercise; 0-time, exercise immediately postprandial; 60-time, exercise one hour postprandial.

[2] Apparent absorptions significantly greater ($P < 0.01$) during control activity; urinary losses significantly different ($P < 0.01$) between diets.

[3] Standard error.

apparent absorption observed during the exercise periods may be a reflection of the inhibitory action of exercise upon gastric secretion (Crandall, '28; Hammar and Öbrink, '53). Exercise may also influence the secretion or re-absorption of metabolic fecal calcium thereby resulting in apparent differences in absorption.

The lack of significant differences in calcium retentions between control and activity periods was contrary to observations in growing dogs by Pinkos,[5] and in old-aged dogs by Liu and McCay ('53) and Wanner[6] in which calcium retention was increased during exercise. Undoubtedly, the major factor concerned is the age difference between the dogs used in the above studies and those in the present study. Differences in intensity and duration of exercise also may be implicated. Hellebrandt and Miles ('32) noted that gentle exercise before or after a meal augmented gastric acidity whereas exhaustive work decreased the acidity. Therefore, the level of exercise may establish the predominance of one response over the other, namely, increased gastric secretion tending to favor calcium absorption and decreased secretion tending to disfavor absorption (McCay, '52).

It becomes evident, then, that the incidence of exercise in relation to time of the meal as well as the level of exercise are essential parameters to consider in calcium metabolism, requiring further study. Finally, mention should be made of the supposed ability of skeletal surfaces to "loosely" adsorb calcium, at least in man (Reifenstein, '54). Accordingly, physical activity, in addition to stimulating osteoblastic activity, may modify the amount of adsorption and retention onto the skeletal surfaces, the degree of modification being dependent upon the intensity and/or duration of the exercise.

No apparent diet and exercise interrelationships were observed in this study. Although in retrospect, an additive effect upon calcium utilization might have been expected by the introduction of exercise to the high-fat diet. This relationship of fat to calcium absorption perhaps deserves further discussion. It is widely known and accepted that fat decreases calcium absorption by the formation of insoluble soaps in the intestine. Another possible mode of action which is rarely mentioned is through the inhibitory effect of dietary fat upon gastric secretion (Kosaka and Lim, '30) which would tend to decrease the absorption of calcium as mentioned earlier. Normally, however, this adverse effect may be ameliorated in part by the concomitant inhibition of gastric motility when fat is ingested (Quigley et al., '34).

The results of this study and an earlier report (Konishi and McCay, '60) indicate that exercise and availability of drinking water are significant variables that should be considered in the interpretation and evaluation of calcium requirements when estimated by chemical balance studies.

SUMMARY

Balance studies were conducted using adult male dogs to investigate the effect of exercise on nutrient utilization with particular reference to calcium. The animals were exercised on a motor-driven treadmill immediately after and one hour subsequent to consuming diets relatively high in either protein, fat or carbohydrate.

A highly significant decrease in the apparent absorption of calcium was observed during the exercise periods. Urinary excretions and retentions of calcium, phosphorus or nitrogen were not significantly different during the exercise. The utilization of dry matter, ether extract and carbohydrate also were not influenced by the level and duration of exercise imposed in this study. There were no apparent diet and exercise interrelationships.

The physiological responses to exercise and their probable effects upon calcium utilization are briefly discussed as well as the relationship of fat in the diet to calcium absorption.

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The authors are indebted to Mrs. Ruth Heeger for assistance with some of the chemical analysis and to Dr. C. R. Henderson for assistance with the statistical analysis of the data.

[5] See footnote 3, p. 125.
[6] See footnote 4, p. 125.

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The Antithyrotoxic Factor of Liver

IV. ACTIVITY OF VARIOUS PURE AND CRUDE MATERIALS

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Hyperthyroidism increases the requirements for a number of nutrients. This series of papers (Overby et al., '59a, b, c) has outlined a method of assay for dietary protective substances using thyrotoxic rats. A comparison of the activity of liver residue with fats and proteins was reported. Over an extended period we have also tested many other crude materials and pure compounds for antithyrotoxic activity. Some of these were tested by others prior to and subsequent to our tests. In the interest of conciseness we chose not to make each test the subject of a separate communication. We condense herein the results of 61 experiments with 28 separate substances. The tests were designed to establish the potency of liver residue for promoting growth and survival of thyrotoxic rats, and to test known compounds and crude materials that might have "borderline" nutritional effects or protect in any way against hyperthyroidism.

EXPERIMENTAL

The techniques for assay were described by Overby et al. ('59a). Ten 21-day-old male Sprague-Dawley rats were used in each experimental group. The basal diets were composed in percentage of casein, 30; sucrose, 57.5; cottonseed oil, 5; salts, 4 (Jones and Foster, '42); inert ingredients, 3.25; choline chloride, 0.1; and vitamins, 0.15. In the present experiments two diets were used, 10–13 and 14. They were identical except that diet 14 contained 50 mg of procaine penicillin per kg.

In each test, three control diets were used: (1) the basal diet, (2) the negative control (basal + 0.35% iodinated casein), and (3) the standard (basal + 0.35% of iodinated casein + liver residue at a level of either 5 or 10%). The test groups received the negative control diet with the test material replacing an equal weight of sucrose.[1]

For presentation of results, the experiments are divided into three series:

1. Thirty-five experiments, testing 17 substances using diet 10–13 and 5% of defatted liver residue as a standard.
2. Twenty-two experiments testing 11 substances using diet 14 and 10% of defatted liver residue as a standard.
3. Six experiments using diet 14 to test reserpine.

Weight gain and survival were the measures of protective activity. Liver residue promoted both. It was assumed that other materials would give a similar response if the same metabolic deficiency was corrected. For estimating activity a relative potency was calculated as follows:

$$\text{Relative Potency} = \frac{A - B}{C - B} \times 100$$

A = product of weight gain and percentage survival of test group;
B = product of weight gain and percentage survival of negative control;
C = product of weight gain and percentage survival of standard group.

The potency of the standard liver residue would thus be 100. These scores agreed well with subjective ratings of the gains and survivals. A potency of 25 or less was not considered as significant activity.

RESULTS

Series 1 experiments. The results are shown in table 1. Comparison of the response elicited by the test materials with the control groups suggests definite activity for distillers' solubles, penicillin mycelia

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[1] We are grateful to the following for supplying materials before they were available commercially: F. M. Strong, University of Wisconsin; M. S. Dunn, University of California, Los Angeles; F. R. Van Abeele, Eli Lilly and Co.; and Russell Rasmusen, Hiram Walker and Sons, Inc.

TABLE 1

Comparative antithyrotoxic activity of various materials using diet 10–13[1]

No. of Experiments	Diet treatment	% of diet	4-Week results		Relative potency[7]
			Av. gain	Surviving	
			gm	%	
		Controls			
35	Basal 10–13		154 ± 7.9[2]	85 ± 5.9	
35	Negative control[3]		60 ± 5.9	36 ± 4.1	
35	Standard[3,4]		92 ± 4.8	77 ± 1.0	
		Test materials[3,5]			
3	Alfalfa meal	5	76	60	44
4	Forage juice	9	75	70	63
1	Distillers' solubles	5	70	60	41
2	Distillers' solubles	10	99	80	112
1	Vitamin B_{13} concentrate	—[6]	53	20	0
3	Herring fish solubles	6	47	30	0
4	Lipoic acid concentrate	—[6]	69	40	12
1	Milorganite	5	47	70	22
1	Defatted pancreas	10	—	00	0
5	Commercial penicillin residue	5	88	80	100
5	Commercial penicillin residue	10	94	70	90
1	Synthetic medium penicillin residue	7.5	88	80	100
1	Defatted spleen	10	73	40	14
2	Brewers' yeast	10	75	30	2
3	Torula yeast	10	119	50	78
1	Adenine + guanine	0.2	52	40	0
1	Orotic acid	0.1	44	20	0
3	Lyxoflavin	0.003	42	70	14

[1] Overby et al. ('59a).

[2] Standard error = $\sqrt{\Sigma d^2/n(n-1)}$.

[3] Diet contained 0.35% of iodinated casein (Protamone, Cerophyl Laboratories, Kansas City, Missouri).

[4] Diet contained 5% of defatted liver residue.

[5] Sources of materials as follows: alfalfa meal and forage juice, Cerophyl Laboratories, Kansas City, Missouri; distillers' solubles and vitamin B_{13} concentrate, Hiram Walker and Sons, Inc., Peoria, Illinois; fish solubles, British Columbia Packers, Ltd., Vancouver, British Columbia; lipoic acid concentrate, Eli Lilly and Co., Indianapolis; Milorganite, Miner Laboratories, Chicago; defatted pancreas, The Armour Laboratories, Chicago; penicillin residues, Abbott Laboratories, North Chicago, Illinois; defatted spleen, The Wilson Laboratories, Chicago; Torula yeast, Lake States Yeast Corp., Rhinelander, Wisconsin; adenine, guanine and orotic acid, Nutritional Biochemicals Corp., Cleveland; lyxoflavin, Merck Sharp and Dohme, Rahway, New Jersey; brewers' yeast, Standard Brands Inc., New York.

[6] Equivalent to 10% of liver residue in the diet.

[7] See experimental section of text.

residue and Torula yeast. The commercial penicillin residue was derived from a growth medium composed of crude and semipurified ingredients. A residue derived from a medium composed of purified and synthetic materials was as active as the commercial product. Borderline activity was indicated for alfalfa meal and forage juice at the levels tested. Adenine, guanine, orotic acid, lyxoflaxin, vitamin B_{13} concentrate, lipoic acid, Milorganite, pancreas, spleen and brewers' yeast were considered inactive in these tests.

Series 2 experiments. The results of the tests with 11 other substances are shown in table 2. Aminoethylisothiouronium bromide appeared to stimulate growth and survival. The optimum level was about 0.2%, but this gave only moderate protection when compared with liver residue. The response to bioflavonoids was inconsistent. There appeared to be better survival in certain cases. Analysis of the entire group of experiments revealed no significant effect due to these compounds. Commercial penicillin residue which had appeared active using diet 10–13 (table 1) was also active using diet 14 which contained 50 mg of procaine penicillin per kg

of diet. However, the relative potency decreased to about 60%.

Reserpine. These experiments (table 3) tested the following: (1) the effect of dietary reserpine without and with iodinated casein and (2) the effect of liver residue in reducing growth inhibition caused by a toxic level of reserpine, with and without iodinated casein. In the absence of iodinated casein, reserpine appeared to have no effect on growth below about 0.65 mg % of the diet. The 2-mg % level inhibited growth, and this growth inhibition was not restored by simultaneous feeding of liver residue.

In the presence of iodinated casein, low, non-toxic levels of reserpine promoted growth and survival. The protection in all cases was much less than with the standard 10% of liver residue. The borderline toxic level (0.65 mg %) of reserpine gave no increased survival or growth of the thyrotoxic animals. The combined toxicity of iodinated casein and 0.65 mg% of reserpine was not overcome by feeding liver residue.

DISCUSSION

Dietary deficiences of thyrotoxic animals may be multiple and overlapping because of the generalized increased body tissue catabolism. Lack of uniformity in published reports of active materials doubtless reflects this multiplicity of deficiencies as well as the diversity of testing procedures. In the present experiments a wide variety of pure compounds and potential sources of unidentified nutrients have been tested. The following discussion summarizes the background of the substances and presents

TABLE 2

Comparative antithyrotoxic activity of various materials using diet 14[1]

Diet treatment	% of diet	5-Week results[2]		Relative potency[7]
		Av. gain	Surviving	
	Controls	*gm*	%	
Basal 14		210 ± 5.1[3]	100 ± —	
Negative control[4]		128 ± 5.5	42 ± 7.5	
Standard[4,5]		189 ± 5.3	99 ± —	
	Test materials[4,6]			
Commercial penicillin residue	10	163	80	57
Defatted thymus	10	111	40	0
Adenosine	0.1	146	10	0
DL-α-Hydroxycaprylic acid	0.02	81	10	0
6-Furylaminopurine	0.008	157	20	0
β-Aminopropionitrile fumarate	0.05	175	10	0
Aminoacetonitrile·H_2SO_4	0.1	107	10	0
Bioflavonoids[8]	0.1–0.5	116 ± 6.6	54 ± 16.5	7
Aminoethylisothiouronium bromide	0.05	154	40	7
Aminoethylisothiouronium bromide	0.1	168	50	23
Aminoethylisothiouronium bromide	0.2	161	90	68
Aminoethylisothiouronium bromide	0.4	155	60	29
Aminoethylisothiouronium bromide	0.8	72	70	0

[1] Overby et al. ('59a).

[2] Mean values for 22 experiments for the controls, 8 experiments for bioflavonoids and 1 experiment each for the other test materials.

[3] Standard error; see footnote 2, table 1.

[4] Finished diet contained 0.35% iodinated casein (Protamone, Cerophyl Laboratories, Kansas City, Missouri).

[5] Finished diet contained 10% of defatted liver residue.

[6] Source of materials as follows: penicillin residue, β-aminopropionitrile fumarate, aminoacetonitrile·H_2SO_4 and aminoethylisothiouronium bromide, Abbott Laboratories, North Chicago, Illinois; defatted thymus, The Wilson Laboratories, Chicago; DL-α-hydroxycaprylic acid, Max S. Dunn, University of California, Los Angeles; 6-furylaminopurine, F. M. Strong, University of Wisconsin, Madison, Wisconsin; bioflavonoids, Sunkist Growers, Ontario, California.

[7] See experimental section of text.

[8] Eight experiments using quercetin, rutin and hesperidin methyl chalcone alone and in combination at levels of 0.1 to 0.5%.

TABLE 3

Summary of 6 experiments testing reserpine with and without iodinated casein[1]

Diet treatment	5-Week results			
	Av. gain	S.E.[2]	Surviving	S.E.
	gm	*gm*	*%*	*%*
Controls				
Basal 14	221	± 8.4	100	—
Negative control[3]	129	± 11.8	22	± 8.6
Standard[3,4]	201	± 8.2	100	—
Reserpine				
0.3 mg/100 gm	229		100	
0.65	216		100	
1.00	206		100	
2.00	190		100	
2.00 + liver residue, 10%	190		100	
Reserpine[3]				
0.01 mg/100 gm	—		0	
0.03	131		30	
0.05	164		60	
0.15	145		80	
0.30	161		60	
0.65	139		10	
1.00	117		40	
2.00	All animals dead in two weeks			
0.65 + liver residue, 10%	172		90	

[1] Protamone, Cerophyl Laboratories, Kansas City, Missouri.
[2] Standard error between experiments $= \sqrt{\Sigma d^2/n(n-1)}$.
[3] Finished diet contained 0.35% iodinated casein.
[4] Finished diet contained 10% of defatted liver residue.

a rationale for their possible relation to thyrotoxicosis.

Sources of unidentified nutrients

Liver residue and unsaturated fats are the most consistently active sources of protection for thyrotoxic rats. Liver and fish by-products, distillers' dried solubles, whey, alfalfa, yeast and fermentation products have been reported as sources of unidentified chick-growth factors. (Lillie et al., '53; Combs, '51; Menge et al., '52; Fisher et al., '54; Edwards et al., '53; Combs et al., '54; Schaefer et al., '55). In the present experiments distillers' dried solubles and penicillin mycelia residue appeared to be almost as active as liver residue in promoting growth and survival of thyrotoxic rats. Alfalfa, forage juice and Torula yeast were less active than liver residue. Fish solubles, Milorganite and brewers' yeast were inactive at the levels tested.

Others have tested several of the above mentioned materials in thyrotoxic rats. Ershoff ('47), Betheil et al. ('47) and Stevens and Henderson ('58) reported that brewers' yeast exerted some beneficial effects, but less than liver residue. O'Dell et al. ('55) attributed no antithyrotoxic activity to brewers' yeast. In our experiment, three different lots of Torula yeast showed somewhat less activity than an equal amount of liver residue.

Antibiotic residues were active in the experiments of Ershoff ('50), and crystalline antibiotics were inactive. Comparison of the activities of penicillin residue and liver residue is difficult because of the known antithyrotoxic effects of low levels of antibiotics (Overby et al., '59a; Meites, '51; Vogel et al., '58). Our experiments show that penicillin residue was as active as liver residue in a diet without antibiotics. With a diet containing 50 mg of procaine penicillin per kg less activity was observed (table 2). The commercial penicillin medium is composed of crude and semipurified ingredients so that a suggested "antithyrotoxic factor" might be present in the medium and not be due to synthesis by the organism. However, it was found that mycelia from penicillin grown in a medium of pure and known compounds

were as active as the comermcial product (table 1).

The experiments with alfalfa and forage juice were carried out prior to the publications of Tappan et al. ('53) and Ershoff et al. ('59). In view of their findings that alfalfa meal must be fed at 20% of the diet for good activity, our level of only 5% was perhaps too low. We did not test the water-insoluble portion of alfalfa, but we did find that the soluble portion (forage juice) was as active as alfalfa meal on a dry weight basis.

Distillers' dried solubles appeared to have unequivocal activity (table 1). It was of interest that orotic acid and a vitamin B_{13} concentrate, both found in distillers' solubles, had no antithyrotoxic activity. Both have produced growth responses in depleted chicks and rats (Manna and Hauge, '53; Rabbi et al., '56). O'Dell et al. ('55) found no activity for orotic acid in thyrotoxic rats.

At the time of the series 1 experiments lipoic acid had not been identified (Reed et al., '53). A concentrate of lipoic acid prepared from liver residue gave no response. Subsequently, Stevens and Henderson ('58) tested a "nutrient mix" supplying 25 mg of DL-lipoic acid per kg of diet. They attributed no activity to the lipoic acid portion of the mix. O'Dell et al. ('55) also tested thioctic acid at 1 mg per kg of diet and reported no activity.

In addition to liver residue, mammalian muscle is reported to be an excellent source of protective materials for thyrotoxic rats (Tappan et al., '53). Pancreas, spleen and thymus tissue were found inactive in our tests. Ershoff ('50) found very little activity for thymus. Stevens and Henderson ('58) reported spleen to be almost as effective as liver residue. Due to the antithyrotoxic activity of dietary unsaturated fats (Overby et al., '59b) differences in activities of animal tissues might be due to the amount and type of fat in the material actually tested.

Purine-like compounds

There have been numerous tests of growth promotion by purine-like compounds. Ershoff ('48) reported no effect on growth of hyperthyroid rats using xanthine, hypoxanthine, adenine and guanine. Xanthine did appear to prolong survival. Huff and Bosshardt ('52) found that dietary adenine, guanine, xanthine, hypoxanthine and the ribosides adenosine, guanosine and inosine partially prevented growth retardation in mice receiving succinylsulfathiazole. Barnett et al. ('56) found that adenosine, isolated from penicillin fermentation residue, was a growth factor for chicks. In our experiments, adenine, guanine, adenosine and orotic acid had no favorable effects on thyrotoxic rats.

Kinetin (6-furylaminopurine) markedly promotes cell division in various plant tissues, and occurs in yeast and other natural materials (Miller et al., '56). In our tests there was no antithyrotoxic activity for kinetin when fed at 0.0083% of the diet.

Emerson and Folkers ('51) reported that lyxoflavin counteracted the growth-depressing effect of thyroid powder in rats. Lyxoflavin was inactive in our tests. Subsequently, Ershoff ('52), Graham et al. ('52) and O'Dell et al. ('55) reported a lack of antithyrotoxic activity for lyxoflavin.

Bioflavonoids

The bioflavonoids are reported to have a diversity of physiological and biochemical actions (Scarborough and Bachrach, '49; Willaman, '55). They may exert biological effects in part through action on the pituitary-adrenal axis (Masri and DeEds, '58) or potentiation of the effects of epinephrine (Wilson and DeEds, '49). Many of the metabolic changes of thyrotoxicosis may not be due directly to the activity of the thyroid hormones, but rather to the increased effects of adrenaline and noradrenaline (Brewster, '56). Leduc et al. ('55) found that the feeding of iodinated casein appeared to interfere in a complex way with the metabolism of catechol amines. D'Iorio and Plaut ('52) found that the addition of thyrotoxic materials to the diet reduced the epinephrine content of adrenals of rats. More recently, Zile and Lardy ('59) showed that the plasma of thyrotoxic rats had about twice as much epinephrine and norepinephrine as plasma from normal rats.

Berezovskaya ('56) found that a catechin preparation "inhibited the hyperfunctioning of the thyroid gland caused by a diet containing iodized casein." Thus there

appeared to be a rationale for testing bioflavonoids for antithyrotoxic activity. The results, as shown in table 2, did not suggest protective activity for rutin, quercetin or hesperidin methyl chalcone. The thyrotoxic effects of iodinated casein were not potentiated in the presence of bioflavonoids.

Hydroxy acids and amino nitriles

Camien and Dunn ('54) found that certain D-α-hydroxy fatty acids are required for growth of certain microorganisms. The D-isomers occur in some natural materials. In our one test with DL-α-hydroxycaprylic acid at 0.0083% of the diet no antithyrotoxic activity was found.

β-Aminopropionitrile and aminoacetonitrile have profound biochemical effects in producing lathyrism in rats (Bachhuber et al., '55). Thyroxine and triiodothyronine alleviate much of the deleterious effect of acetonitrile (Ponseti, '57). In our experiments neither of the nitriles, at levels not toxic to normal rats, favorably affected rats made thyrotoxic with iodinated casein. At higher levels the two toxicities were additive. Liver residue did not protect the animals against the nitrile toxicity.

Radioprotective agents

Liver residue has been found to inhibit radiation lethality in rats (Ershoff, '54). The protective factors were not found in thymus and spleen. Thiols and related compounds, particularly cysteamine and aminoethylisothiouronium bromide (AET), minimize injury from X-irradiation of mammals (Pihl and Eldjarn, '58). These compounds also have antithyroid action in rats (Osmer et al., '59). In our experiments, AET produced both growth promotion and survival of thyrotoxic rats. The maximum effect, at about 0.2% of the diet, was much less than that given by 10% of liver residue. Higher levels of AET caused anorexia.

Reserpine

Our interest in reserpine in thyrotoxicosis arose from the report of Ford and colleagues ('53), showing clinical improvement in hyperthyroid patients treated with reserpine. There appeared to be little effect on the raised oxygen consumption of the thyrotoxic patient. Canary and colleagues ('57) have also found the drug to be useful in thyrotoxicosis without depressing thyroid activity. Other reports indicate that reserpine has a direct antithyroid effect in animals. These reports were reviewed by Ershoff ('58) who tested high levels of reserpine in thyroid-fed rats. He found that doses of reserpine and thyroid which when given individually were nonlethal, rapidly resulted in death when given concurrently. Moon and Turner ('59a, b) studied the effect of reserpine on thyroid activity in rats, and suggested that reserpine alters thyroid function by inhibiting thyrotropin secretion.

Our experiments indicate that reserpine at levels below about 0.3 mg per 100 gm of diet increases growth and survival of rats receiving iodinated casein. At about 0.65 mg % reserpine was slightly toxic alone. At this level and above, the toxic effects of the two materials appeared to be additive. Liver residue had no effect on reserpine toxicity. It appeared that the mechanism of protection by liver residue was different from that by reserpine and AET.

SUMMARY

The activity of liver residue in promoting growth and survival of thyrotoxic rats was compared with known compounds and crude materials for which there is some rationale for possible protective effects. Of 5 sources of unidentified chick factors tested, only distillers' dried solubles and penicillin residue were as active as liver residue. Alfalfa and Torula yeast showed some activity but less than liver residue. No activity was observed for purine-like compounds and bioflavonoids. D-α-Hydroxycaprylic acid, β-aminopropionitrile and aminoacetonitrile were inactive. Aminoethylisothiouronium bromide and reserpine showed protective activity, but less than was shown by liver residue.

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Phosphorylated Vitamin D_2 and Alkaline Phosphatase Activity

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The activity of alkaline phosphatase in blood plasma is greatly increased in rickets (King, '53). Robison ('23) has observed that the phosphatase activity of bone was higher in rickets. DeLuca and Steenbock ('56) have studied the action of vitamin D on the plasma phosphatase activity using diets differing widely in calcium and phosphorus content. In these studies the amount of phosphatase in plasma could not be correlated with the severity of rickets. Prophylactic or therapeutic administration of vitamin D reduced the values.

Higher values for plasma phosphatase activity have also been observed in various forms of bone diseases not related to vitamin D deficiency (King, '53). In general, the alkaline phosphatase activity of plasma and bone increases when there is an increase in the osteoblast activity.

Administration of vitamin D results in an increase in the blood level of calcium (Carlsson, '52), and in increased absorption of calcium from the intestinal tract (Nicolaysen et al., '53). Vitamin D appears also to have a local effect in the bones which differs from the effect exerted on the calcium metabolism and thereby secondarily on the phosphate metabolism, and is necessary for normal development of the skeleton (Nicolaysen and Eeg-Larsen, '53).

Robison and Rosenheim ('34) advanced the theory that the alkaline phosphatase in the bone is responsible for a local increase in the concentration of inorganic phosphate and thereby facilitates the deposition of bone salt as the solubility product is exceeded. Due to this possible action of alkaline phosphatase in the mechanism of calcification, this enzyme has attracted much attention, and several hypotheses for its significance have been proposed.

Zetterström and Ljunggren ('51) claimed that the addition of phosphorylated, water-soluble vitamin D_2 (D_2P) to an incubation medium resulted in activation of the activity of alkaline phosphatases from various sources. The results of this investigation have received some attention as a possible mechanism for the mode of action of vitamin D. We have therefore felt that a restudy of this problem was necessary.

EXPERIMENTAL

Determination of enzyme activity. The phosphatase activity was determined according to Bessey et al. ('46). As substrate 0.0075 M disodium *p*-nitrophenylphosphate was used, prepared according to the method of Bessey and Love ('52). To this, 0.001 M magnesium sulfate was added. The various buffers used are given in the description of the experiments. All concentrations given refer to the final concentration in the incubation mixture. Adjustment of pH was made with dilute hydrochloric acid or dilute sodium hydroxide to pH 10.0 using a pH meter.

All incubations were carried out at 37°C in test tubes. The final volume was 1 ml. The enzyme solution was added to the test tubes after 10 minutes of equilibration. Preliminary investigations revealed that the enzymes were stable during the first 10 minutes of incubation, but that during the next 20 minutes some denaturation took place. The rate of hydrolysis was initially of zero order.

In some experiments 0.006 M disodium phenylphosphate[1] was used as substrate. The end point was determined by analyses of the liberated phenol according to Buch and Buch ('39).

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[1] Merck.

Preparation of enzymes. Purified intestinal alkaline phosphatase was prepared from dog feces according to Armstrong ('35).

Alkaline phosphatase from bone was purified according to the following procedure: metatarsals and metacarpals from newly killed calves were obtained from a slaughter house. The bones were cleaned of all adhering tissues and chopped to small cubes. The bone shaft with the bone marrow was discarded. The pieces were extracted with acetone for three hours at 0°C, dried overnight at room temperature, and ground to a coarse powder. The acetone extraction was repeated for three hours at −20°C. The insoluble material was filtered off and dried. The powder could then be stored for several months at room temperature without loss of enzyme activity. The yield was 500 gm of powder from 20 bones.

One half of the powdered bone, 250 gm, was extracted three times with isotonic potassium chloride at 0°C, each time for 24 hours. The insoluble was filtered off and ammonium acetate/ammonium hydroxide buffer was added until the buffer concentration was 0.1 M and pH was 8.9. All subsequent operations were carried out at 0°C.

Small portions of bentonite suspended in water were added until the supernatant was clear after centrifugation. An excess of bentonite was avoided. Ammonium sulfate was added to 0.85 saturation. The precipitate was centrifuged off after two hours and discarded. Acetone was added to the supernatant in excess. The active protein fraction separated on the interphase acetone-water after 8 hours and could be collected with a spatula.

The precipitate was taken up into 0.05 M ammonium acetate/ammonium hydroxide buffer, pH 8.9, and dialyzed against running tap water for 8 hours at 7°C. Thereafter acetone was added to 0.6 saturation and the precipitate formed after two hours was discarded. The acetone concentration was increased to 0.7 saturation. The precipitate was collected after 24 hours, dissolved in distilled water and lyophilized. The yield was 500 mg of protein. A twenty-fold purification from the crude extract was obtained in this way.

Phosphorylation of calciferol. Crystalline calciferol was phosphorylated essentially according to Zetterström ('51a). Two-point factorial dose assay (Bliss, '51) was carried out, using an aqueous solution of the disodium salt of D_2P.[2] The content of biologically active vitamin D was 41.9 ± 7.4%. A correction for inactive materials is taken into account when the concentration is given for D_2P; the final concentration of biologically active D_2P in the incubation medium was 5×10^{-5} M.

Chemicals. All chemicals were of analytical grade.

RESULTS

Observations from the experiment carried out with intestinal alkaline phosphatase are presented in table 1. Four differ-

[2] The authors wish to acknowledge the assistance of Professor R. Nicolaysen in carrying out this assay.

TABLE 1

The activity of intestinal phosphatase using various buffers[1] and the effect of D_2P

Incubation time	0.068 M Veronal buffer		0.068 M Ethanolamine buffer		0.068 M Glycine buffer		0.068 M Tetraborate buffer		
	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$	Increase
minutes									%
2.5	0.038[2]	0.034	0.038	0.041	0.038	0.033	0.006	0.023	300
5	0.073	0.071	0.076	0.078	0.073	0.073	0.013	0.039	200
7.5	0.105	0.102	0.116	0.111	0.110	0.109	0.023	0.053	128
10	0.145	0.142	0.147	0.145	0.143	0.140	0.040	0.069	73
20	0.258	0.254	0.276	0.280	0.244	0.240	0.075	0.103	38
30	0.327	0.320	0.385	0.382	0.342	0.334	0.111	0.124	12

[1] One microgram of purified intestinal phosphatase was added to each tube after 10 minutes' equilibration. The incubations were carried out at 37°C. The substrate was 0.0075 M disodium *p*-nitrophenyl phosphate; 0.001 M magnesium sulfate was added. The total volume was one milliliter. the pH 10.0; 5×10^{-5} M D_2P added when required.

[2] The values are micromoles of *p*-nitrophenol liberated.

ent buffers were used: veronal, ethanolamine, glycine and tetraborate buffer. The buffer concentrations were the same in all cases, 0.068 M. The activity of the enzyme was the same in the first three buffers in the order mentioned, and additions of D_2P did not affect the enzyme activity. With the tetraborate buffer the rate of hydrolysis was only 29% of the rate obtained using the other systems. The initial activity was increased many fold following the addition of D_2P to the tetraborate system, and successively it was reduced to the rate of the control experiment.

The experiments were repeated using a purified phosphatase from bone. The results are given in table 2. The enzyme activity was only slightly lowered using the tetraborate buffer; the activity was also slightly lowered using the veronal buffer as compared with the glycine and ethanolamine buffers. Additions of D_2P did not affect the enzyme activity.

Additional observations were made using a crude extract of rat kidney. A weighed amount of fresh tissue was homogenized in water at 0° using a glass homogenizer of the conventional Potter Elvehjem type. The homogenate was diluted to 50 ml with water, and 25 µl was added to each test tube. The results are given in table 3. The tetraborate buffer did not inhibit the enzyme in this case, although a slight increase of the initial activity was observed in this buffer when D_2P had been added. In the other buffer systems tested, D_2P failed to produce any such increases.

These experiments indicated that the effect obtained when D_2P was added, was the result of a partial reversal of the inhibitory action exerted by the tetraborate buffer. The effect was most pronounced

TABLE 2

The activity of bone phosphatase using various buffers[1] and the effect of D_2P

Incubation time	0.068 M Veronal buffer		0.068 M Ethanolamine buffer		0.068 M Glycine buffer		0.068 M Tetraborate buffer	
	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$
minutes								
2.5	0.025[2]	0.024	0.024	0.018	0.028	0.029	0.023	0.029
5	0.051	0.051	0.048	0.044	0.055	0.052	0.044	0.047
10	0.089	0.088	0.093	0.087	0.118	0.109	0.089	0.089
20	0.156	0.153	0.189	0.185	0.204	0.189	0.160	0.171
30	0.214	0.216	0.276	0.262	0.262	0.262	0.233	0.229

[1] Two hundred micrograms of bone phosphatase were added to each tube after 10 minutes' equilibration. The incubations were carried out at 37°C. The substrate was 0.0075 M disodium *p*-nitrophenyl phosphate; 0.001 M magensium sulfate was added. The total volume was 1 ml, and the pH of the medium was 10.0; 5×10^{-5} M D_2P (final concentration) was added.

[2] The values are micromoles of *p*-nitrophenol liberated.

TABLE 3

The activity of kidney phosphatase (crude extract)[1] using various buffers and the effect of D_2P

Incubation time	0.068 M Veronal buffer		0.068 M Glycine buffer		0.068 M Tetraborate buffer		
	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$	Increase
minutes							%
2.5	0.043[2]	0.045	0.039	0.040	0.039	0.052	33
5	0.074	0.079	0.074	0.076	0.077	0.094	22
10	0.156	0.153	0.144	0.147	0.152	0.163	7
20	0.269	0.273	0.240	0.247	0.269	0.275	2
30	0.367	0.371	0.334	0.341	0.374	0.371	0

[1] Fresh kidney, 0.722 gm, was taken from a stock rat, homogenized and diluted with water to 50 ml; 25 µl of this extract were added to each tube, after 10 minutes' equilibration at 37°C. The substrate was 0.0075 M *p*-nitrophenyl phosphate; 0.001 M magnesium sulfate was added, the total volume being 1 ml, and pH, 10.0. When required, D_2P was added to a final concentration of 5×10^{-5} M.

[2] The values represent micromoles of *p*-nitrophenol liberated.

with alkaline phosphatase from the intestine, and this enzyme was therefore selected for the following experiments.

Two different tetraborate buffers were used: 0.002 M tetraborate-0.018 M sodium carbonate, and 0.068 M tetraborate buffer. The control experiment was carried out in ethanolamine buffer (0.068 M). The results are reported in table 4. The activity of the intestinal phosphatase was greatly reduced in the 0.068 M tetraborate buffer, and in the 0.002 M tetraborate-0.018 M sodium carbonate system the enzyme activity was also lower than in the control experiment. Additions of D_2P again failed to increase the activity in the control system whereas such an increase was observed in the tetraborate buffers. The greatest increase of the activity was observed in the 0.068 M tetraborate buffer.

One experiment was carried out in a system identical to that of Zetterström and Ljunggren ('51) except that 0.001 M magnesium sulfate was added. Buch and Buch's ('39) modification of King and Armstrong's method for the determination of phosphatase activity was used. The substrate was 0.006 M disodium phenyl phosphate, and the incubations were carried out in 0.068 M veronal or a 0.002 M tetraborate-0.018 M sodium carbonate buffer. The pH was 10.0. Two micrograms of the intestinal enzyme were added to each tube making a total volume of 5 ml. The results are given in table 5. In both buffers the addition of D_2P resulted in an initial increase of the rate of hydrolysis, with the greatest increase obtained using the tetraborate buffer.

Additions of D_2P had failed previously to affect the enzyme activity when a veronal buffer was used. This could have been due to the change either in the substrate or in the analytical procedure used for determin-

TABLE 4

The effect of D_2P upon the activity of intestinal phosphatase in relation to the concentration of tetraborate[1]

Incubation time	0.068 M Ethanolamine buffer		0.068 M Tetraborate buffer			0.002 M Tetraborate—0.018 M sodium carbonate buffer		
	$-D_2P$	$+D_2P$	$-D_2P$	$+D_2P$	Increase	$-D_2P$	$+D_2P$	Increase
minutes					%			%
2.5	0.049[2]	0.049	0.009	0.025	178	0.034	0.049	44
5	0.071	0.078	0.019	0.039	105	0.065	0.082	26
7.5	0.103	0.109	0.031	0.049	58	0.097	0.119	23
10	0.145	0.152	0.044	0.065	48	0.135	0.152	13
20	0.255	0.255	0.089	0.115	29	0.236	0.262	11
30	0.349	0.349	0.136	0.149	10	0.327	0.345	6

[1] The experimental conditions were identical to those given in table 1.
[2] The values are micromoles *p*-nitrophenol liberated.

TABLE 5

The activity of intestinal phosphatase in a system according to Zetterström and Ljunggren ('51) as compared with the activity in 0.0068 M veronal buffer[1]

Incubation time	0.002 M Tetraborate—0.018 M sodium carbonate buffer			0.068 M Veronal buffer		
	$-D_2P$	$+D_2P$	Increase	$-D_2P$	$+D_2P$	Increase
minutes			%			%
2.5	5.9[2]	12.5	112	5.9	9.3	58
5	14.7	22.0	50	13.5	18.0	34
10	38.2	53.5	40	32.0	37.0	16
20	70.0	89.0	27	63.5	69.5	9
30	109.0	128.0	18	102.5	108.0	6

[1] The substrate was 0.006 M sodium phenyl phosphate to which 0.001 M magnesium sulfate was added. Two micrograms of intestinal phosphatase were added to each tube after 10 minutes' equilibration. The incubations were carried out at 37°C. The pH of the medium was 10.0, the total volume being 5.0 ml. D_2P was added to give a concentration of 5×10^{-5} M.
[2] The values are micrograms of phenol liberated.

ing the enzyme activity. Furthermore, it was expected that D_2P would be hydrolyzed by the enzyme. The phenol reagent of Folin and Ciocalteu is well known to produce a non-specific color with a variety of substances (Hawk et al., '54).

Incubation of 5×10^{-5} M D_2P was carried out in a system identical to that previously described (table 5), but with no substrate added. Phenol determinations were made and the color was developed in the usual way. The intensity corresponded to that of 10 μg of phenol after 10 minutes' incubation. When the reaction was stopped after 5 minutes' incubation, the color corresponded to that of 7.7 μg of phenol. Water suspensions of calciferol will also produce a color, as well as emulsions of calciferol in "Tween 60," although the intensity of the obtained color varies considerably.

DISCUSSION

The results demonstrate that the effect of D_2P reported by Zetterström and Ljunggren ('51) cannot be confirmed in experiments carried out under similar conditions. Addition of D_2P resulted in an increased enzyme activity when tetraborate buffer was used.

The intestinal activity of intestinal phosphatase was inhibited in this buffer; and this observation agrees well with that of Zittle and Della Monica ('50) who have reported that tetraborate competitively inhibits alkaline phosphatase from the intestine.

Bone phosphatase was not inhibited by the tetraborate, and additions of D_2P failed to increase the initial rate of hydrolysis when this enzyme was used. Using a crude extract from rat kidney, addition of D_2P increased the initial rate of hydrolysis slightly in the tetraborate buffer, although this buffer failed to inhibit the enzyme activity.

It is not clear why additions of D_2P may have an effect with the tetraborate buffer, although it is possible that surface activation may be responsible. In any case, the effect is an artifact and does not appear to have any relationship to the mode of action of vitamin D.

The activation of D_2P reported by Zetterström and Ljunggren ('51) is a composite effect due to the tetraborate buffer and the analytical method employed in the determinations of enzyme activity.

With regard to the effect reported by Zetterström ('51b) of D_2P on the aerobic oxidation in kidney mitochondria, it was found in experiments which have not been reported that the observation of Zetterström ('51b) could not be confirmed. With regard to the effect of vitamin D on the oxidation of citrate, reference is made to the papers of DeLuca et al. ('57a, b) who noted lower rates for citrate oxidation in kidney homogenates or kidney mitochondria taken from rats treated with prophylactic doses of vitamin D compared with vitamin D-deficient controls.

SUMMARY

The effect of phosphorylated vitamin D_2 (D_2P) on the activity of alkaline phosphatases which had been partially purified from intestine and bones, and a crude extract from kidney, has been investigated. Additions of D_2P to a final concentration of 5×10^{-5} M did not affect the activity of these enzymes when the determinations were carried out under conditions for optimal enzyme activity. Various buffers have been used in these investigations. Addition of D_2P resulted in a partial reversal of the inhibitory action exerted by tetraborate on the intestinal alkaline phosphatase. Furthermore, D_2P was found to be hydrolyzed by alkaline phosphatase from the intestine. Free calciferol thus liberated will give a color with the phenol reagent of Folin and Ciocalteu.

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A Fat-Soluble Material in Alfalfa that Reduces the Biological Availability of Tocopherol[1]

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After analyzing farm feeding materials for their tocopherol content, Brown ('53) reported that grasses, clover and alfalfa had a high tocopherol content, 95 to 100% of which was α-tocopherol. Work performed in this laboratory (Singsen et al., '55) showed that the tocopherol of alfalfa was not fully available to the chick when the prevention of encephalomalacia was used as the criterion of response. In a report based upon that work, it was stated that only about 25% of the tocopherol, as measured chemically, was utilized by the chick. Also reporting from this laboratory, Bunnell ('57), using another chick bioassay method in which he found that the response measured as liver tocopherol varied linearly with the intake, stated that approximately one-third of the tocopherol of alfalfa was available to the chick. Pudelkiewicz et al. ('60) confirmed the linearity of this relationship and used the chick bioassay to determine the relative potencies of natural and synthetic α-tocopherol and their acetate esters.

Eaton et al. ('58) measured the tocopherol content of 5 tissues in each of 40 Holstein calves after the vitamin E-depleted animals had been fed either dehydrated alfalfa leaf meal or *d*,α-tocopheryl acetate. When the over-all percentage availability of the 5 tissues had been considered, it was tentatively suggested by these authors that the chemically determined total tocopherol in alfalfa was not completely available to the calf. Availability of the averaged unweighted percentages, however, was approximately 90%.

Scott and Nelson,[2] on the other hand, compared the feeding of 6.25 and 12.5% of alfalfa meal, which represented 5.8 and 11.6 I.U. of vitamin E per pound of diet, with 4.1 and 5.8 I.U. of vitamin E per pound of diet as α-tocopheryl acetate in the feeding of chicks. He reported that the vitamin E activity of alfalfa meal, based upon the prevention of exudative diathesis, was the same as that determined chemically. It is well known that the alfalfa plant absorbs selenium quite readily and that selenium prevents exudative diathesis in chicks (Patterson et al., '57; Schwarz et al., '57); hence, the vitamin E activity reported by Scott may have been due to the combined effect of both tocopherol and selenium.

A sequence of studies has been made in an attempt to account, in part, for the "unavailability" of the tocopherol of alfalfa to the chick and is presented in this publication.

EXPERIMENTAL

Day-old White Plymouth Rock male chicks were fed the Connecticut vitamin E-low diet (Singsen et al., '54) for 13 days. The basal diet contained the following percentage of ingredients: white corn meal, 68.3; soybean oil meal (50% protein), 10; crude casein, 15; fish meal (50 to 60% protein), 2.5; dicalcium phosphate, 2; limestone, 1.5; sodium chloride, 0.50; manganese sulfate, 0.0125; and vitamin mix, 0.15. In experiments 119F and 119G, 2% of white corn meal was replaced by 2% of a tocopherol-low specially prepared coconut oil[3] during the supplementation period to act as a carrier for the pure tocopherol oils. The smallest and largest chicks were

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[1] This work was supported in part by grants from the Yantic Grain and Products Company, Norwich, Connecticut.
[2] Scott, M. D., and T. S. Nelson 1956 Vitamin E in chick nutrition. Federation Proc., *15:* 572 (abstract).
[3] This is a specially prepared refined, bleached and deodorized, tocopherol-low and sterol-free coconut oil, Product 1223 of the E. F. Drew and Company, Inc., Boonton, New Jersey.

then removed and the approximately 60% remaining were distributed into groups of 8 chicks each. Each of these groups, approximately of equal weight, was placed on a tocopherol-supplemented diet for two weeks. There were two non-supplemented groups in each of the experiments. Each treatment was supplemented on an equimolar basis at levels equivalent to 8 and 16 mg of tocopheryl acetate per pound of diet. In order to obtain 8- and 16-mg levels of tocopherol by the addition of alfalfa to the diet, it was necessary to add approximately 7.5 and 15%, respectively, of a high potency alfalfa leaf meal.

In all other diets, including the basal, alfalfa was replaced by a mixture of white corn meal, solvent-extracted soybean oil meal and α-cellulose in a ratio of 10:7:3. This mixture had approximately the same protein and fiber content as alfalfa leaf meal. Tocopherol analyses of alfalfa, feed, excreta and liver tissue were made by extraction of a sample with hot absolute ethanol, molecular distillation and chromatographic separation through a column of Florex XXS. Methods used have been described in detail previously (Pudelkiewicz et al., '60). Basal diets and diets supplemented with the tocopherols were routinely analyzed to insure proper supplementation. The tocopherols used in experiment 119 through 119E were in the form of fine gelatin-sugar beadlets, and the tocopherols used in experiments 119F and 119G were pure oils. Beadlets were used in preference to the pure oils in most of the experiments because of the ease of weighing and mixing a dry product into the diets.

RESULTS AND DISCUSSION

Feeding the vitamin E-low diet for 13 days reduced the liver tocopherol content of the chicks from approximately 150 to 2 to 3 μg/gm of tissue. The slope ratios, presented in table 1, were obtained over a three-year period using three different samples of alfalfa leaf meal with potencies of 190, 223 and 218 μg of tocopherol per gram. These ratios showed that the approximate one-third availability of vitamin E from alfalfa was obtained whether or not plasma or liver was used to measure the response. The slope ratio was obtained by dividing the slope which is the regression of the response (micrograms of tocopherol per gram of tissue) on intake (milligrams of tocopherol added as tocopheryl acetate per pound of diet) when alfalfa was the source of tocopherol by the slope obtained when either pure *d,α*-tocopherol or *d,α*-tocopheryl acetate was the source. A slope ratio of 1.00 would, of course, indicate equal utilization. Similar slope ratios were obtained when alfalfa was compared with either pure *d,α*-tocopherol or its stable acetate ester, indicating that destruction of the alcohol form in the gut was probably not the cause for the apparent lack of utilization.

It was further hypothesized that perhaps the free tocopheryl, as found naturally in alfalfa, was partially destroyed by the time the feed was consumed. Since tocopheryl acetate is stable, the comparison was made only between the diets containing alfalfa and those containing *dl,α*-tocopherol beadlets. Feed was analyzed prior to and at the end of the experiment. During the two-

TABLE 1

Comparison of the availability of d,α-tocopherol of alfalfa to pure d,α-tocopherol and d,α-tocopheryl acetate expressed as slope ratios and based upon the tocopherol content of plasma and liver tissue

Experiment	Plasma		Liver	
	Alfalfa *d,α*-tocopheryl acetate	Alfalfa *d,α*-tocopherol	Alfalfa *d,α*-tocopheryl acetate	Alfalfa *d,α*-tocopherol
119	—	—	0.37	—
119B	0.39	—	0.33	—
119C	0.40	—	0.34	—
119D	0.35	0.32	0.33	0.33
119E	0.43	0.46	0.38	0.40

week supplementation period that the diets remained at temperatures of approximately 24 to 27°C, there was little, if any, destruction of tocopherol in either of the diets.

The green color of the excreta from chicks that were fed the diets containing alfalfa suggested the advisability of balance studies. From a known quantity of feed intake, all the excreta of a 48-hour period, comprising 12 separate collections, were stored at −20°C immediately after removal from glass collecting plates. The excreta were then dried by lyophilization and analyzed for tocopherol. Results, expressed as percentage excretion of *added* tocopherol, are presented in table 2. Experiments 119D and 119E represent the results of two experiments in which tocopherol, supplied as alfalfa, was compared with the standards *d,α*-tocopheryl acetate and *d,α*-tocopherol, both fed as fine gelatin beadlets. It is readily apparent that the excreta from chicks that ate the diets including alfalfa contained a much larger amount of tocopherol than the excreta from those chicks fed the standards. Experiment 119D, which was run in January, showed that there was approximately twice as much excretion of the tocopherol from alfalfa-fed chicks as from those chicks fed the purified forms; however, in experiment 119E, run in August, there was approximately three times as much excretion.

Since all diets used in these experiments had approximately the same fiber content, it was thought that the high level of tocopherol excretion by the alfalfa-fed birds was due primarily to the close association of the tocopherol with the fibrous particles of alfalfa which were not readily digested in the chick, and then to the fact that it takes only as little as three hours for food to pass through the digestive tract of the chick. To determine whether or not the fibrous nature of alfalfa tied up the tocopherol, alfalfa was extracted with hot ethanol in a soxhlet apparatus, the extract concentrated by removing part of the solvent, the concentrate analyzed for tocopherol and added to diets at the equivalent levels of 8 and 16 mg of tocopheryl acetate. The feed in this experiment (119F) and the next (119G) was stored immediately after mixing and during the experimental period at −20°C. Results, shown in table 2 (experiment 119F), are again expressed as percentage excretion of *added* tocopherol. It is obvious that the removal of the tocopherol, along with the other complex lipids from the fibrous material of the alfalfa, did nothing to enhance utilization; excretion of the tocopherol by chicks that had either alfalfa or the extract of alfalfa in their diets was approximately the same. Excretion of tocopherol by the chicks that had either alfalfa or the lipid extract in their diets was again approximately twice that of the pure tocopherols. It was felt, at this point, that something in the lipid portion of alfalfa must influence the utilization of the tocopherol.

With this assumption in mind, alfalfa lipids were separated into three fractions and were added to diets at levels that would normally be encountered if alfalfa were fed at levels equivalent to the 8 and 16 mg of tocopheryl acetate per pound. The first step in obtaining these fractions was, again, the extraction of the lipids from alfalfa with hot ethanol. Molecular distillation of the extract left essentially a tocopherol-low residue and a distillate which contained the tocopherol, plant ster-

TABLE 2

Percentage excretion of added tocopherol

Supplement	Experiment					
	119D February		119E August		119F October	
	8	16	8	16	8	16
	mg equivalent tocopheryl acetate per pound of diet					
	%	%	%	%	%	%
d,α-Tocopheryl acetate	20.15	19.85	14.22	12.41	24.43	27.81
d,α-Tocopherol	32.83	24.03	10.38	11.69	22.69	26.14
Alfalfa leaf meal	47.52	55.80	35.30	43.50	41.06	54.84
Alfalfa extract	—	—	—	—	45.54	56.72

TABLE 3
Percentage excretion of added tocopherol (experiment 119G)

Supplement	mg equivalent tocopheryl acetate per pound of diet	
	8	16
	%	%
d,α-Tocopheryl acetate[1]	23.2	23.9
d,α-Tocopherol[1]	28.7	31.9
d,α-Tocopherol[1] + sterols[2]	38.8	27.7
Alfalfa	64.5	66.0
Tocopherol distilled from alfalfa	25.1	36.7
d,α-Tocopherol[1] + tocopherol-low alfalfa lipids	61.7	64.3

[1] Pure tocopherol oil.
[2] Sterols were precipitated from the molecular distillate of alfalfa.

ols, some carotene and, undoubtedly, other unknown substances.

Upon analysis of the residue, it was found that in amounts which represented the quantity of alfalfa required to give the equivalent of 8-and 16-mg levels of tocopheryl acetate, the residue contributed 0.2 and 0.4 mg, respectively, of tocopheryl acetate per pound of diet. Most of the sterols were removed by low temperature precipitation from ethanol. The remainder of the distillate was analyzed for tocopherol and added to diets to give the equivalent of 8- and 16-mg levels of tocopheryl acetate per pound of diet. The three extracts were dissolved in ethanol prior to mixing into diets. An equal amount of ethanol was also added to the basal diets and those containing alfalfa leaf meal. Results of this experiment are presented in table 3. The most obvious fact to be observed from these data is that approximately 60% of the pure *d*,α-tocopherol which was added to the tocopherol-low alfalfa lipids was excreted; the percentage excretion was approximately equal to that of the leaf meal itself. Another interesting fact to be observed from these data is that the *d*, α-tocopherol of alfalfa, when separated from the rest of the lipid matter, was not excreted to any appreciably greater extent than the pure tocopherols. Sterols did not appear to affect the excretion of tocopherol.

When this laboratory first reported that the vitamin E from alfalfa was poorly utilized, it was suggested that the chemical analysis used for determining tocopherol in alfalfa was in error. This is possible when one considers the fact that a commercial grade of alfalfa meal, besides containing vitamin K, has been shown by Lester and Crane ('59) to contain approximately 100 μg of Q_{254} and approximately 10 μg of coenzyme Q_{10} per gm of dry alfalfa. These fat-soluble compounds, when in the reduced state, react with the $FeCl_3$-bipyridine reagents and would be attributed to tocopherol unless they were removed. If the value obtained for total tocopherol by chemical analysis were due partly to vitamin E and partly to these other reducing substances, it is conceivable that a lower utilization would be realized. Since the amount of tocopherol in the liver is a good indicator of the α-tocopherol content of a diet and has been used as the criterion of response in a chick bioassay, the amount of tocopherol in the liver of chicks that consumed the experimental diets would show the effects of the treatments more reliably. Observations of the tocopherol content of liver are presented in table 4. Alpha-tocopheryl ace-

TABLE 4
Tocopherol content of liver tissue, micrograms/gram (experiment 119G)

Supplement	mg equivalent tocopheryl acetate per pound of diet		
	0	8	16
		μg/gm	
d,α-Tocopheryl acetate[1]	2.4	8.2	13.8
d,α-Tocopherol[1]	2.2	8.1	14.0
d,α-Tocopherol[1] + sterols[2]	—	8.1	13.5
Alfalfa	—	5.5	5.7
Tocopherol distilled from alfalfa	—	8.0	13.1
d,α-Tocopherol[1] + tocopherol-low alfalfa lipids	—	6.9	11.1

[1] Pure tocopherol oil.
[2] Sterols were precipitated from the molecular distillate of alfalfa.

tate and *d,α*-tocopherol are the standards. The sterols that were precipitated from the molecular distillate of alfalfa showed that they had no influence on the pure *d,α*-tocopherol which was added to the diet. Alfalfa showed the typical low liver tocopherol, and as pointed out previously, this was due, at least in part, to excretion of tocopherol. When the tocopherol distillate from alfalfa, or in other words, the natural *d,α*-tocopherol which was separated from the alfalfa lipids was fed, values that would indicate a utilization of better than 90% were obtained. That 100% utilization was not achieved could possibly be due to the fact that the distillate contained compounds in addition to tocopherol which were not removed and which could, perhaps, be the cause of some interference in absorption. The high utilization of this tocopherol, separated from alfalfa, also indicated that the method of assay used in this laboratory for tocopherol in alfalfa is valid; that is, tocopherol is measured and no appreciable amounts of other reducing substances that are present in alfalfa. In fact, Pudelkiewicz and Matterson ('60) have recently shown that molecular distillation, when used as a step in the determination of tocopherol, removed the interference due to coenzyme Q_{10}. No explanation can be made for the fact that the tocopherol-low alfalfa extract caused a high excretion of added tocopherol, but not as low a liver tocopherol as obtained when alfalfa was fed; although the several prolonged heat treatments to which the lipid extract was subjected may have caused sufficient alteration of key constituents to account for this.

SUMMARY

1. The *d,α*-tocopherol in alfalfa is only about one-third available to the chick. It was discovered that a compound or compounds in the hot ethanol extract of alfalfa act antagonistically to tocopherol, increasing its excretion and decreasing its availability.

2. The vitamin E of alfalfa, when separated from the rest of the meal, is fully available.

3. The addition of pure *d,α*-tocopherol to a tocopherol-low lipid extract of alfalfa resulted in an excretion of tocopherol to the same extent as that of the tocopherol excretion when alfalfa was the source of *d,α*-tocopherol.

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The Mechanism of the Thiamine-Sparing Effect of Penicillin in Rats[1]

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Although thiamine-sparing action of several antibiotics, including penicillin, has been firmly established, the mechanisms of this action have not been solved. Guerrant and Steel ('58) have reported a lowering of the thiamine concentration in the cecum and feces as a result of antibiotic feeding and concluded that the antibiotic increased the absorption of thiamine from the gut. Mameesh and Johnson ('58), on the other hand, have shown that the amount of available thiamine supplied by the intestinal microflora was increased by penicillin. They concluded that the effect of penicillin could not be accounted for by an increase in the intestinal absorption of thiamine. The present studies were designed to investigate this controversial question.

Male weanling rats (Holtzman, Wis.) were used throughout these studies. They were maintained for three days on a complete purified diet, then divided into groups so as to equalize weight distribution. Rats were caged individually in raised wire screen-bottom cages in a room maintained at 72° ± 2° and fed ad libitum. In most experiments coprophagy was prevented by the method of Barnes et al. ('57). The composition of the complete diet is given in table 1. Thiamine was removed from the B-vitamin mixture in the diet of the thiamine-deficient groups, and 50 mg of crystalline procaine penicillin was added per kilogram to the diets of the groups that were to receive penicillin. When fecal thiamine was to be determined, fresh feces were extracted immediately with 0.1 N HCl and the extracts stored at 4°C until ready for analysis. Total thiamine was determined by the method of Hennessy and Cerecedo ('39).

TABLE 1
Diet composition

Major components	
	gm
Casein[1]	25.0
Dextrin	53.0
Hydrogenated vegetable oil[2]	15.0
Salts[3]	4.0
Choline dihydrogen citrate	0.3
B vitamins in sucrose	2.0
Fat soluble vitamins in corn oil	1.0
Total	100.3

B vitamins in 2.0 gm sucrose	
	mg
Thiamine·HCl	0.40
Riboflavin	0.80
Pyridoxine·HCl	0.40
Ca pantothenate	4.00
Niacin	4.00
Inositol	20.00
Biotin	0.02
Folic acid	0.20
Vitamin B_{12}	0.03
Menadione	1.00

Fat-soluble vitamins in 1.0 gm corn oil	
	mg
Vitamin A acetate	0.31
Vitamin D (calciferol)	0.0045
α-Tocopherol	5.00

[1] Vitamin-Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Primex, Proctor and Gamble, Cincinnati.
[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14*: 273.

RESULTS

Effect of preventing coprophagy on thiamine-sparing by penicillin. Twelve groups of 5 rats each were established. Four groups received the complete diet (adequate thiamine), 4 a diet containing 0.25

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mg of thiamine/kg and in 4 groups the thiamine was removed from the diet completely. Two of each of these groups received penicillin in the diet and at each level of thiamine coprophagy was prevented in one group receiving penicillin and in one group not receiving it. Figure 1 shows the results in the groups not receiving penicillin. Some growth was evident in the conventional rats receiving the thiamine-free diet and those receiving the suboptimal-thiamine diets. The use of dextrin as the carbohydrate source probably enhanced intestinal synthesis to some extent. When coprophagy was prevented, rats in the two thiamine-deficient groups lost weight after the first week and some had died by the 4th week. It is quite evident that the partial maintenance of rats on a dextrin-containing diet without thiamine was completely dependent upon the ingestion of feces.

In figure 2 the results for the groups receiving penicillin are shown. The remarkable sparing effect of penicillin in the conventional rats is clearly demonstrated. However, when coprophagy was prevented anorexia and loss of weight developed quickly. The thiamine-sparing effect of penicillin was obviously dependent upon the ingestion of feces. Comparing figures 1 and 2, in the two low-thiamine groups, the coprophagy-prevented rats receiving penicillin showed slight improvement in

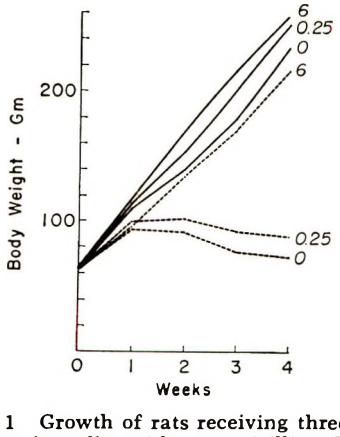

Fig. 1 Growth of rats receiving three levels of thiamine in a diet without penicillin. Solid lines, coprophagy permitted, and broken lines, coprophagy prevented. Numbers at the right are milligrams of thiamine hydrochloride per kilogram of diet.

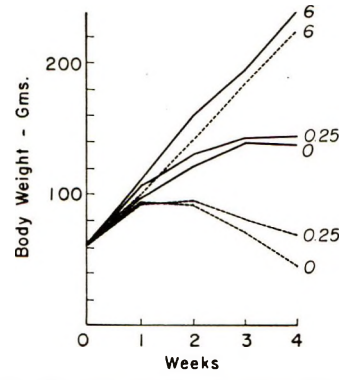

Fig. 2 Growth of rats receiving three levels of thiamine in a diet with penicillin. Solid lines, coprophagy permitted, and broken lines, coprophagy prevented. Numbers at the right are milligrams of thiamine hydrochloride per kilogram of diet.

growth over the conventional controls. Others experiments have demonstrated that, with meticulous attention in the maintenance of the tail cups so as to prevent any access to feces, penicillin did not have any discernible effect upon growth under conditions of limited thiamine intake.

Growth response to graded doses of thiamine with or without penicillin in the diet. Ten groups of 6 rats each were fed a thiamine-free diet, with coprophagy prevented in all rats. Five groups received a diet containing penicillin and 5 were fed no penicillin. A water solution of thiamine hydrochloride was administered daily, 6 times per week, by stomach tube so that groups both with and without penicillin received either zero, 2, 4, 6 or 8 μg of thiamine hydrochloride/day. The growth rates given in figure 3 show that penicillin did not affect the response to a given dose of thiamine over the 4-week test period.

Since penicillin might require the presence of fecal microflora in the upper intestinal tract and this was abolished by preventing coprophagy in the above study, a modification of the conditions for the dose-response experiment was designed. The general condition of feeding a thiamine-free diet either with or without penicillin and the daily administration of zero, 2, 4 or 6 μg of thiamine hydrochloride by stomach tube to groups of 6 rats each was repeated. Fecal collection cups were used on all rats, but this time feces collected in

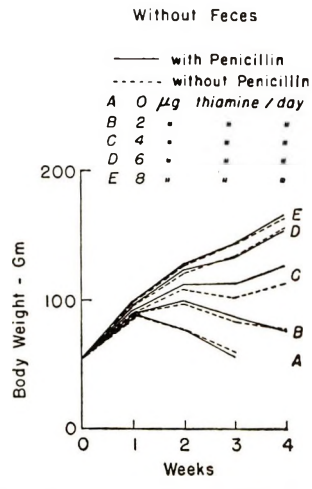

Fig. 3 The effect of penicillin in the diet on growth of rats with coprophagy prevented and fed 5 levels of thiamine by stomach tube.

tail cups from another set of rats were fed in separate feeder jars to the experimental groups. The rats providing these feces received a diet containing 1 mg of thiamine/kg so as to keep them alive during the 4-week test period. Glucose[2] was used as the source of carbohydrate so as to reduce the amount of fecal thiamine. Feces collected in tail cups from each rat were fed to a corresponding rat that was receiving one of the graded doses of thiamine by stomach tube. The results shown in figure 4 provide evidence that penicillin did not affect growth rate even in the presence of constant reinoculation of the upper intestine with fecal organisms. It is evident that a low level of thiamine was carried over with the feces from the donor rats since the growth curves in figure 4 are slightly higher than those in figure 3.

These two experiments show clearly that penicillin does not affect the amount of thiamine absorbed from the upper intestine nor does the antibiotic alter tissue requirements.

The effect of penicillin on the amount of thiamine in feces. Six groups of 10 rats each were fed a thiamine-free diet for 4 weeks. Three groups received the diet with penicillin. Thiamine hydrochloride was given by stomach tube daily for 6 days each week at levels of zero, 4 and 8 µg/rat/day to groups fed or not fed penicillin. All rats were prevented from eating their feces. During the third week, feces were collected daily for 7 days and each day's collection was pooled for the rats in a given group for total thiamine analysis. At the end of the 4th week the rats were killed and livers from each group were pooled for thiamine analyses. Results are presented in table 2 and a graphic representation of the effect of penicillin on the total fecal output of thiamine is shown in figure 5. It is evident that penicillin does increase the intestinal synthesis and total fecal output of thiamine. However, these results do not prove whether the increase in fecal thiamine is sufficient to account for the increased growth rate that is exhibited by conventional rats receiving suboptimal thiamine in a diet supplemented with penicillin. The following, rather complicated study, was designed to test this hypothesis.

Determination of the proportion of fecal thiamine available to the rat and the effect of penicillin on the amount of available thiamine. Four groups of 10 rats each were fed the thiamine-free diet for a 4-week period. Tail cups for the prevention of coprophagy were used on all rats. The groups, which were designated A, B, C and D were treated as follows. The feces from the rats in group A were collected daily

[2] Cerelose.

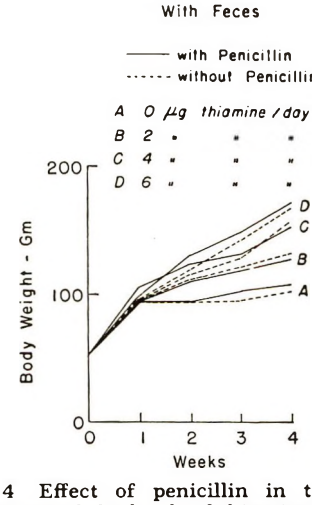

Fig. 4 Effect of penicillin in the diet on growth of rats fed 4 levels of thiamine by stomach tube. Natural coprophagy was prevented, but feces from another group of rats not receiving penicillin were fed separately from the diet.

TABLE 2

Total thiamine in feces and liver as influenced by the presence of penicillin in the diet; averages per rat per day for 7 daily fecal collections taken during the third week on the experimental regimen

Thiamine·HCl[1] intake by stomach tube	Body weight at end of period	Food intake during period	Weight of feces	Fecal thiamine	Liver weight	Liver thiamine
μg/day	*gm*	*gm*	*gm*	*μg*	*gm*	*μg/gm*
			Without penicillin			
0.0	69	5.4 ± 0.34[2]	0.51 ± 0.05	6.1 ± 0.29	2.68	0.62
3.4	129	9.5 ± 0.65	1.23 ± 0.12	6.0 ± 0.21	4.93	0.90
6.9	177	14.2 ± 0.66	1.64 ± 0.04	5.8 ± 0.29	7.59	1.02
			With penicillin			
0.0	78	5.9 ± 0.47	0.48 ± 0.06	7.8 ± 0.34	2.83	0.58
3.4	144	10.7 ± 0.90	1.16 ± 0.05	8.6 ± 0.32	5.44	0.89
6.9	186	14.3 ± 0.75	1.53 ± 0.08	9.1 ± 0.33	7.65	1.08

[1] Dosage by stomach tube 6 times each week. Average daily intake is 6/7 times actual dosage.
[2] Standard error of the mean.

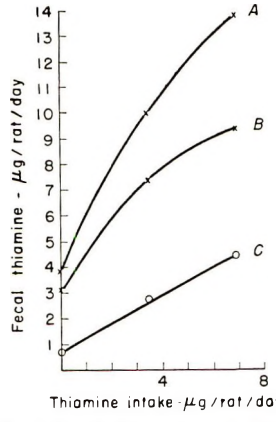

Fig. 5 Total fecal thiamine at three levels of thiamine intake as affected by penicillin in the diet. A, penicillin included in the diet; B, no penicillin in the diet; C, difference between A and B.

from the tail cups and placed in a separate feeder jar in the same rats' cage. No penicillin was included in the diet. The feces from rats in group B were collected daily from the tail cups and fed back to the same rats. This group received penicillin in the diet. The diet of group C included no penicillin, but that of group D did. The feces from group D rats were fed to the rats in group C, and the feces of group C rats were fed to the rats in group D. Body weights were recorded weekly for 4 weeks. For calculations to be described later, the three-week body weights were of primary importance. The amount of feces consumed was determined by collecting spilled fecal pellets on the papers under the cages together with feces remaining in the special feeder jars. This was done daily and the pooled daily non-ingested feces for each group were dried at 100° for 24 hours and weighed. During one 24-hour period in both the third and 4th weeks the collection of feces from the tail cups was not fed back, but was dried and weighed. From these data the percentage of fecal output eaten was calculated.

Total thiamine was determined on 24-hour pooled samples of feces from the tail cups at the end of the third and 4th weeks. By this scheduling there was only one day during the first three weeks that rats did not receive feces.

Simultaneously with the above experiment, 4 groups of 10 rats each, receiving a thiamine-free diet without penicillin were given daily (6 days per week) doses of thiamine hydrochloride by stomach tube, either zero, 2, 4 or 8 μg/rat/day. The weight gain at the end of the third week was plotted against the log of the average daily dosage of thiamine.

From these data it was possible to interpolate the thiamine required in order to account for the three-weeks' growth found in the 4 groups of rats being fed feces. Since the average fecal output and percentage of ingestion had been estimated and the total thiamine in these feces had been determined, the total fecal thiamin consumed each day could be calculated

The growth curves provided an estimate of the actual "available" thiamine consumed on an average for each day. Comparing the total ingested thiamine with the total available provided a means of comparing the percentage of "available" thiamine for the different groups. The presence of penicillin in the diet did not influence the availability of the fecal thiamine (table 3). This percentage is remarkably low, averaging about 35% of the total. In planning this experiment it was hoped that some information would be obtained from groups C and D regarding the effect of ingesting penicillin while the rats were eating feces from a rat that did not receive penicillin, and vice versa. However, the results indicate that some penicillin must have been excreted in the feces and that when these feces were ingested, the penicillin acted qualitatively in the same manner as penicillin in the diet. No other explanation seems to account for the increased growth rate of rats in group D.

DISCUSSION

The abolishment of the thiamine-sparing effect of penicillin when coprophagy is prevented confirms the earlier report of Mameesh et al. ('59). Coprophagy also appears to account for the growth response of rats receiving thiamine-free diets containing dextrin. These observations, together with the recent studies showing the lack of thiamine-sparing by antibiotics in germ-free rats (Wostmann et al., '58) lead to the following conclusions: (1) the intestinal microflora is essential to this vitamin-sparing effect; (2) coprophagy is essential for the utilization of thiamine that has been synthesized in the intestinal tract; (3) thiamine is not absorbed from the lower intestine; and (4) no significant quantity of free thiamine can be released in that region of the small intestine where absorption takes place. An accurate reproduction of the phenomenon termed refection as first described by Fredericia et al. ('27) has not been seen in these laboratories. The inclusion of dextrin in the diet as the carbohydrate source permits enhanced growth using thiamine-free or low thiamine diets. This may not be a manifestation of refection; however, the dextrin effect is completely abolished when coprophagy is prevented.

The equal growth response to graded doses of thiamine, with or without penicillin in the diet, rules out the possibility of the antibiotic having any profound effect upon thiamine absorption or tissue requirements. This confirms the conclusion of Mameesh and Johnson ('58) but differs from that of Guerrant and Steel ('58).

TABLE 3

Total fecal thiamine ingested compared with thiamine utilized by the rat; a calculation of "available" fecal thiamine and the effect of penicillin in the diet; data for a group represent feces fed to that group; all rats were fitted with fecal collection cups

	Penicillin treatment		Collection period	3 week body weight gain	Fecal thiamine fed[1]	Feces ingested	Fecal thiamine ingested[1]	Thiamine utilized[1,2]	"Available" fecal thiamine
	Donor	recipient							
				gm	*μg*	*%*	*μg*	*μg*	*%*
A	−	−	1		13.7	83	11.4		
			2		9.7	89	8.6		
			Av.	51	11.7		10.0	3.6	36
B	+	+	1		24.3	86	20.9		
			2		27.0	85	23.0		
			Av.	91	25.7		22.0	7.7	35
C	+	0	1		22.0	79	17.4		
			2		21.7	92	20.0		
			Av.	84	21.9		18.7	6.8	36
D	0	+	1		23.7	71	16.8		
			2		23.3	87	20.3		
			Av.	74	23.5		18.5	5.6	30

[1] Expressed as μg per rat per day for the three-week test period.
[2] Interpolated values from log-dose response of rats receiving graded amounts of thiamine by stomach tube.

Maximal growth rate of rats having coprophagy prevented and receiving an excess of thiamine in the diet (4.0 mg of thiamine HCl/kg of diet) has been observed to be 106 gm of weight gain in three weeks. Extrapolating this weight gain on a log dose-response curve indicates that the daily requirement during this active growth period is approximately 9 μg of thiamine hydrochloride. Mameesh et al. ('56) have estimated the requirement to be approximately one μg of thiamine per gram of diet.

The clear-cut increase in fecal thiamine resulting from penicillin in the diet does not agree with the report of Guerrant and Steel ('58). However, in the present studies, fecal thiamine concentrations showed relatively small variability as compared with the Guerrant and Steel data. Furthermore, the very high percentage of fecal ingestion which thiamine-deficient rats exhibit suggests that any such measurement of fecal concentration may be invalid unless coprophagy is prevented.

It will be noted from table 2 that increase in fecal thiamine resulting from penicillin feeding was the result of concentration in the feces. No increase in weight of feces excreted was observed. On the other hand, graded doses of thiamine increased food consumption and weight of feces excreted. However, the concentration of thiamine was not affected by the level of thiamine fed.

The final experiment in this study provides evidence that the thiamine sparing action of penicillin can be accounted for entirely by the increased thiamine synthesized in the large intestine. Actually, the experiment in which feces were fed back to rats receiving or not receiving penicillin in the diet was conducted twice. The first time, measurements of fecal thiamine were not made, but by interpolating from the curves relating thiamine intake to fecal thiamine, these values could be estimated. Calculations similar to those given in table 3 confirmed the fact that penicillin did not alter the percentage of total fecal thiamine that was available to rats upon ingestion of feces.

It will be noted that the total fecal thiamine found when feces were fed (table 3) was much greater than that obtained when rats were not fed their feces (table 2). This must mean that change in the intestinal flora is brought about by coprophagy. A continuous re-inoculation of the intestinal tract as a result of the practice of coprophagy enhances the total biosynthesis of this vitamin.

Only speculation can be offered in explanation for approximately 65% of fecal thiamine not being available. Some of this thiamine may be in a chemically unavailable form. Viable bacterial cells are not well digested in the stomach or small intestine (Pounden et al., '50). Furthermore, there is some evidence that thiamine synthesized by cecal micro-organisms is confined to the cells and does not diffuse into the medium (Abdal-Salaam and Leong, '38).

SUMMARY

By means of a technique for the complete prevention of coprophagy in the rat it has been possible to study the mechanism by which an antibiotic, penicillin, spares thiamine. The following conclusions have been drawn:

1. Thiamine which is synthesized in the intestinal tract is not directly absorbed, but is utilized only after excretion in the feces, and the ingestion of those feces.
2. No significant quantity of free thiamine is released in that region of the upper intestine where absorption takes place.
3. The sparing of thiamine by penicillin and by certain dietary carbohydrates is abolished when coprophagy is prevented.
4. Penicillin does not alter the extent of thiamine absorption from the intestinal tract nor does it affect tissue requirements.
5. Penicillin in the diet increases the amount of thiamine synthesized in the large intestine and is reflected by an increase in fecal thiamine.
6. This increase in fecal thiamine can account entirely for the growth response observed, since penicillin does not alter the proportion of fecal thiamine available to the rat.
7. Approximately 35% of the total thiamine excreted in the feces is utilized by the rat when the feces are eaten.
8. The minimal thiamine requirement for optimal growth during the first three-weeks' post-weaning in the rat, during

which coprophagy is prevented, has been calculated to be approximately 9 μg of thiamine hydrochloride per day.

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Effect of Six-Day Starvation on Rat Liver Lactic Dehydrogenase Activity[1]

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Withdrawal of nutrition from a normal mammalian results in the stress of sustaining the blood sugar level through the physiological and biochemical mechanisms of the animal itself. The study of the biochemical sequence of events during prolonged starvation is a valuable tool in the elucidation of the metabolic adaptation processes which the organism undergoes during the necessity of maintaining blood sugar level through gluconeogenesis (Weber, '59). Studies of liver enzymes involved in production and disposal of glucose-6-phosphate demonstrated that the various enzymes which metabolize this hexosemonophosphate ester show different behavior (Weber and Cantero, '57a, b; '58). It has become clear that as a result of fasting some of the enzymes decrease parallel with the depletion of liver nitrogen, whereas others are preferentially maintained or preferentially decreased (Weber, '59).

Recently investigation was extended to explore the effect of fasting on biochemical mechanisms involved in pyruvate metabolism. One of the most important pathways of pyruvate utilization is its conversion to lactate by lactic dehydrogenase activity. The work to be reported here describes the effects of starvation on the behavior of rat hepatic lactic dehydrogenase (LDH) during fasting.

MATERIALS AND METHODS

Animals and experimental procedures. Male Wistar rats weighing 180 to 220 gm were used. The animals were maintained on laboratory chow[2] and water ad libitum and kept in separate cages. Fasted animals received only water. The rats were sacrificed one, three and 6 days after the fasting began. Fed animals of the same weight-group were used to obtain normal values. Four or more animals were in each group. No animal died during the 6-day starvation period.

Preparation of liver homogenate and supernatant fluid. The animals were stunned, decapitated and bled. The preparation of homogenates and supernatant fluid (Weber and Cantero, '58) and the determination of liver cellularity (Weber and Cantero, '57c) are described elsewhere.

Biochemical procedures. LDH activity was determined in the supernatant fluid which was prepared by centrifuging liver homogenate for 30 minutes at 0°C at 100,000 × g in a refrigerated Spinco model L centrifuge. The course of the reaction was followed by observing the decrease in optical density at 340 mμ at 30-second intervals for a period of 5 minutes using the Beckman Quartz Spectrophotometer, Model DU, with glass cells no. 2097. The reaction cuvette contained a mixture of the following reagents in final molarity: sodium pyruvate (2.5×10^{-4} M); DPNH (2.5×10^{-5} M); phosphate buffer (0.2 M) pH 7.4; sufficient enzyme to give linear kinetics over a period of 10 minutes at 37°C. Water was added to give a final reaction volume of 3.0 ml. The blank contained all the reagents except DPNH. The assay was started by the addition of the enzyme solution (Weber and Cantero, '59). Activity was expressed in micromoles of pyruvate converted into lactate. Unit of activity is defined as the amount of en-

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[1] Preliminary experiments on this project were carried out by the author in the Montreal Cancer Institute, Research Laboratories, Notre Dame Hospital and the University of Montreal, Montreal, Canada. Part of these data was presented at the First Canadian Liver Carbohydrate Symposium, March 7, 1959, Montreal, Canada (Weber, '59).

[2] Purina Fox Chow, Ralston Purina Company, St. Louis.

zyme which produces one micromole of lactate per hour per gram of wet weight of liver at 37°C. In the calculation of the units, the extinction coefficient for DPNH equal to 6.2×10^3 was used. Enzymatic activities in these studies were expressed *per average cell* and *per 100 gm of body weight*. The microKjeldahl procedure was used for nitrogen determinations.

First, in preliminary kinetic studies, the pH optimum, substrate optimum and proportionality of enzymatic activity with amount added and with reaction time were established. After these criteria were fulfilled, the effect of fasting was examined.

RESULTS AND DISCUSSION

Effect of 6-day fasting on liver constituents. The effect of 6-day fasting on body weight, liver weight, liver/body weight ratio and liver cellularity is presented in table 1. During this period the rats lost 32% of the initial body weight. Liver weight decreased to 39% and the liver/body weight ratio fell to 64% of the value of normal-fed rats. During this starvation period the liver cellularity gradually increased to 191% of the value of the normal-fed rats, indicating a shrinkage of the liver. Comparison of these observations with previous results (Weber and Cantero, '57b, '58) shows that such parameters, which are always registered in starvation studies, are well reproducible. Since the observations agree well with previous results (Weber and Cantero, '57b, '58), they are not discussed here in detail.

The effect of 6-day fasting on hepatic nitrogen content and LDH activity. In table 2 a comparison is made between liver ni-

TABLE 1

Effect of 6-day fasting on liver constituents

	Fed controls	Rats fasted following number of days		
		1	3	6
Initial body weight, gm	202 ± 5[1]	207 ± 5	210 ± 6	208 ± 7
Final body weight, gm	226 ± 10	186 ± 7	159 ± 3	141 ± 5
Loss in weight, %	11	10	24	32
Liver weight, gm	10.0 ± 0.4 (100)[2]	6.3 ± 0.6 (63)	5.2 ± 0.2 (52)	3.9 ± 0.7 (39)
Liver/body ratio × 100	4.4 ± 0.2 (100)	3.4 ± 0.3 (77)	3.3 ± 0.1 (75)	2.8 ± 0.5 (64)
Cellularity[3]	207 ± 17 (100)	291 ± 18 (141)	339 ± 24 (164)	396 ± 18 (191)

[1] Means and standard deviations for 4 or more animals.
[2] Values within parentheses express data in percentage, 100% representing value for control animals.
[3] Expressed in millions of counted nuclei per gram of wet weight of liver.

TABLE 2

Effect of 6-day fasting on liver nitrogen content and lactic dehydrogenase activity

	Fed controls	Rats fasted following number of days		
		1	3	6
Activity/average cell, μmoles $\times 10^{-6}$/cell	72.5 ± 3.4[1] (100)[2]	52.8 ± 4.0 (74)	44.1 ± 4.8 (61)	39.1 ± 3.8 (54)
Nitrogen/average cell, mg $\times 10^{-8}$/cell	7.6 ± 0.8 (100)	5.8 ± 0.4 (76)	5.2 ± 0.5 (68)	4.1 ± 0.5 (54)
Activity/100 gm body weight, μmoles $\times 10^{-6}$/100 gm	323 ± 54 (100)	182 ± 26 (56)	145 ± 19 (45)	110 ± 28 (34)
Nitrogen/100 gm body weight, mg $\times 10^{-8}$/100 gm	33.4 ± 3.0 (100)	19.7 ± 1.2 (59)	17.2 ± 0.9 (51)	11.5 ± 0.9 (34)

[1] Means and standard deviations for 4 or more animals.
[2] Values within parentheses express data in percentage, 100% representing value for control animals.

trogen content and LDH activity during the 6-day starvation period, expressed per cell and per 100 gm of body weight. The nitrogen content of the average liver cell decreased to 76% at one day, to 68% at three days and went down to 54% at the end of 6-days fasting. It is of interest that LDH activity in the average liver cell decreased with the nitrogen content. Such a parallelism between liver nitrogen content and LDH activity was noted also when enzymatic activity was expressed per 100 gm of body weight (table 2).

Previous studies of the behavior of enzymes involved in G-6-P utilization have shown that hepatic glucose-6-phosphatase activity was preferentially maintained well above the decreasing nitrogen level during 6 days of fasting (Weber and Cantero, '58). On the other hand, phosphoglucomutase and phosphohexoseisomerase activities decreased with the decreasing nitrogen content (Weber and Cantero, '58). The data presented here indicate that LDH activity was not preferentially maintained during 6-day starvation, but its behavior was similar to that of hepatic phosphoglucomutase and phosphohexoseisomerase.

The dietary deprivation studies have delineated the range to which liver LDH activity may be depressed under physiological circumstances. It is of interest that in the neoplastic liver LDH activity decreased to 17% as compared with the activity of the average normal liver cell (Weber and Cantero, '59); whereas under physiological conditions no decrease less than 50% could be obtained in the fasted animals.

SUMMARY

The effect of 6-day starvation on hepatic lactic dehydrogenase activity was investigated.

During a 6-day starvation period the rats lost 32% of the initial body weight. The liver/body weight ratio decreased to 64% and the liver weight went down to 39% of the value of normal-fed rats. During this fasting period the liver cellularity increased to 191% of the value for the normal-fed rats, owing to the shrinkage of the liver.

The nitrogen content of the average liver cell decreased progressively, reaching 54% of the values for the fed animals at the end of the 6-day starvation period. Hepatic lactic dehydrogenase activity paralleled liver nitrogen content, reaching a final value of 54% at the end of the fasting. Similar results were obtained when nitrogen content and lactic dehydrogenase values were expressed per 100 gm of body weight.

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Biosynthesis of Sterols and Fatty Acids as Affected by Nicotinic Acid and Related Compounds[1]

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In an attempt to elucidate the mechanism whereby high levels of nicotinic acid (NAc) lower blood cholesterol in certain species including man, Gaylor et al. ('60) compared the effects of high levels of NAc and related compounds on blood cholesterol in the chick and the rat. Isonicotinic acid (INA) produced significant depressions in both species, but NAc depressed blood cholesterol only in the chick and actually caused elevations in the rat; nicotinamide (NAm) or benzoic acid were without marked effect on blood cholesterol. Changes in liver pyridine nucleotides, liver cholesterol or bile acids or fecal sterols, when they occurred at all did not appear to be related to the effects of the niacin analogues on blood cholesterol.

The present studies deal with alterations in hepatic cholesterol and fatty acid synthesis under the influence of NAc and related compounds. Limited studies on effects of NAc on the incorporation of acetate into sterols have been reported by Merrill ('58) and by Perry ('59). Merrill ('58) found that rats fed high levels of NAc incorporated more of an injected dose of acetate-1-C^{14} into liver sterols *in vivo*, and the addition of NAc to liver slices from unsupplemented rats also produced marked increases in the incorporation of the labeled acetate into sterols. On the other hand, Perry ('59) reported that the addition of large amounts of NAc suppressed the incorporation of acetate into both sterols and fatty acids.

EXPERIMENTAL

Feeding of NAc and analogues. Male Holtzman rats weighing about 100 gm received ad libitum a basal diet having the following percentage composition: casein, 18; sucrose, 67; lard, 10; Wesson salts ('32), 4; vitamin mixture,[4] 0.1; choline chloride, 0.1; vitamin E solution (50 mg vitamin E/ml), 0.09; and halibut liver oil, 0.09; 0.1 or 1% of NAc or its analogues were added to the experimental diets at the expense of sucrose.

Barred Plymouth Rock chicks were given a basal diet having the following percentage composition: sucrose, 56; casein, 23; gelatin, 10; salts V, 6; soybean oil, 4; feeding oil (300 I.U. vitamin A, 60 I.U. vitamin D_3 and 0.3 mg vitamin E/100 gm diet), 1; L-cystine, 0.3; and water-soluble vitamins[5] with 1% of NAc or its analogues added to the experimental groups at the expense of sucrose. In some cases the chicks received a basal diet in which 10% of lard or 10% of lard plus 0.5% of cholesterol replaced all of the soybean oil and some of the sucrose. The diets were fed for 4 weeks, and at suitable intervals, animals were decapitated and liver slices prepared freehand for incubation with $CH_3C^{14}OONa$.

Addition of NAc and analogues. Liver slices from adult rats previously maintained on the Steenbock stock ration were added to the buffer containing various con-

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[3] P.H.S. Research Fellow of the National Cancer Institute.
[4] The vitamin mixture consisted of the following in milligrams: inositol, 100; Ca pantothenate, 20; niacin, 10; menadione, 10; riboflavin, 2; thiamine·HCl, 2; pyridoxine·HCl, 2.5; biotin, 0.1; folic acid, 0.2; vitamin B_{12}, 0.02; and glucose to 1 gm.
[5] The following vitamins (in milligrams) were added per 100 gm of diet: thiamine·HCl, 0.6; riboflavin, 0.9; Ca pantothenate, 2.0; niacin, 5.0; pyridoxine·HCl, 0.8; biotin, 0.03; inositol, 100; choline chloride, 200; folic acid, 0.2; vitamin B_{12}, 0.002; and menadione, 0.02.

centrations of NAc, NAm, INA, DPN,[6] and TPN[7] in which the excess acidity had previously been neutralized with KOH.

Incubation of liver slices with acetate. One gram of liver slices was incubated at 37°C for three hours, unless otherwise indicated, in 6 ml of Krebs-Hensleit bicarbonate buffer containing 1.33 μc (0.24 μM) of acetate-1-C^{14} (Brooks and Baumann, '57). This is about 5 to 10% of the substrate used by some others (Hill et al., '58). The incubation was stopped by the addition of 6 ml of ethanol and the mixture was saponified for 6 hours with 2 gm of KOH. Sterols were extracted from the cooled mixture with Skellysolve A and the residue remaining after evaporation was dissolved in acetone-ethanol and precipitated with digitonin. The digitonide was washed with ether, dried, and split with anhydrous pyridine, and the sterols extracted into anhydrous ether. An aliquot of sterol was assayed for cholesterol and a suitable portion of the remainder dissolved in a 0.5% solution of polyvinylpyrollidone in chloroform, and 0.5 ml plated onto one-inch copper planchets for counting under a shielded G-M tube. When livers from animals fed NAc or its analogues were studied, the results were expressed as total activities per gram of liver, since the specific activities reflected a bias due to changes in liver cholesterol levels resulting from the dietary treatments. Fatty acids were extracted and fractionated for counting as described previously (Gaylor et al., '60). In unincubated control liver slices, all of the counts remained in the acidified aqueous fraction after the extractions; no activity was found in either the sterol or fatty acid fractions.

Pyridine nucleotides. Total oxidized pyridine nucleotides were determined by the method of Feigelson et al. ('50).

RESULTS

Changing rates of incorporation of acetate into sterols by liver slices from rats fed NAc or related compounds. Rats were fed the lard diet plus 0.1 to 1.0% of NAc, NAm or INA for one to 28 days and the liver slices incubated with $CH_3C^{14}OONa$. The control values for the incorporation of acetate into sterol varied from day to day (table 1). However, the values for individual incubations were less variable; namely, the mean specific activity of the 28-day control value was 912 with a standard error of 90.5. This variation parallels the experience of others (Bucher et al., '59).

In general the feeding of NAc or NAm depressed the incorporation of acetate into sterol for several days after which there was an enhanced rate of incorporation followed by a return to normal. Thus after one to three days of treatment, one per cent of NAc produced depressions of 12, 47 and 44% of the control values, respectively, whereas 1.0% of NAm produced depressions of 43, 75 and 79% of the control values respectively (table 1). Lower concentrations of NAc or NAm produced lesser depressions of sterol synthesis at this time; one per cent of INA produced no significant alteration. The marked initial decrease in incorporation resulting from feeding NAm may have been associated with a decreased food intake and resultant weight loss in these animals. No significant alteration in either food consumption or growth was noted with the other compounds fed nor with lower levels of NAm. After one per cent of NAc had been fed for 14 days, the rate of incorporation into sterol was increased by 120% over the control rate (table 1), and after 28 days the rate returned to normal. NAm increased incorporation substantially on the 7th day, and by 291% on the 14th day; at 28 days the rate also returned to normal. Rats fed INA on the other hand showed a marked increase in sterol synthesis on the 28th day. Thus both the direction and the extent of the alteration in sterol synthesis appeared to depend upon the length of time the rats had been fed NAc, NAm or INA. Our 14-day results agree with the conclusion of Merrill ('58) who observed an increase of 83% with the incorporation of injected acetate into sterols by rats fed 0.8% of NAc for 8 days.

Relative changes in biosynthesis of sterols and fatty acids by liver slices. Liver slices from rats fed NAc, NAm or INA for

[6] Diphosphopyridine nucleotide, 96% by assay from Sigma Chemical Co., St. Louis.

[7] Triphosphopyridine nucleotide from Pabst Laboratories, Milwaukee.

TABLE 1

Incorporation of acetate-1-C^{14} into sterols by liver slices from rats fed nicotinic acid or related compounds for various intervals

Diet	Days on diet												
	1		2		3		7		14		28		
	Total counts	% of Control	Total	%	Total	%	Total	%	Total	%	Specific activity	Total	%
	c/min/gm[1]		*c/min/gm*[1]		*c/min/gm*[1]		*c/min/gm*[1]		*c/min/gm*[1]		*c/min/mg*[2]	*c/min/gm*[1]	
Control (10% lard)	1210[3]	100	1710	100	935	100	576	100	432	100	912[4]	1560	100
Control (10% lard) + 0.1% nicotinic acid					665	72							
Control (10% lard) + 0.5% nicotinic acid					476	51							
Control (10% lard) + 1.0% nicotinic acid	1060	88	900	53	525	56	443	77	950	220	742	1635	105
Control (10% lard) + 0.1% nicotinamide					913	98							
Control (10% lard) + 0.5% nicotinamide					414	44							
Control (10% lard) + 1.0% nicotinamide	685	57	428	25	201	21	795	138	1690	391	1074	1460	94
Control (10% lard) + 0.1% isonicotinic acid					614	66							
Control (10% lard) + 1.0% isonicotinic acid					885	95					1730	2910	186

[1] Counts/min/gm of liver-slices incubated.

[2] Counts/min/mg of sterol.

[3] Each value represents the average of 4 separate incubations, each incubation representing one to three rats.

[4] Standard error of the mean = ±90.5; coefficient of variability = 32.8%. The variations in control values at the other times were similar as were the variations in the groups given nicotinic acid. Bucher et al. ('59) reported similar variations among control values for acetate incorporated into cholesterol by microsomal preparations.

three weeks were incubated with labeled acetate and the incorporation into both sterols and fatty acids determined. With all these compounds an increase in sterol synthesis was associated with a decrease in fatty acid synthesis (table 2), and the magnitude of the increases into sterols frequently appeared to vary with the magnitude of the decrease in fatty acid synthesis (table 2, lines 2-5). This inverse relationship was not, however, an absolute one, and there also appeared to be effects associated with the specific nicotinic analogue employed. Thus the two levels of INA tested lowered fatty acid synthesis by about 6000 counts whereas the increase in sterol was never more than 1100.

A reciprocal relationship between fatty acid synthesis and sterol synthesis was also suggested in a series of experiments with livers from chicks fed various analogues of NAc for 4 weeks. Livers from control chicks fed 4% of soybean oil showed a high rate of fatty acid synthesis (73,000 c/m/gm of liver) and a low rate of sterol synthesis, 592 c/m (table 3). NAc, NAm

TABLE 2

Incorporation of acetate-1-C^{14} into sterols and fatty acids by liver slices from rats fed NAc or related compounds for three weeks

Treatment	Incubations[1]	Incorporation			
		Sterol		Fatty acids	
		Total counts	%	Total counts	%
	no.	*c/min*		*c/min*	
10% Lard	4	2400	100	10,660	100
10% Lard + 0.1% nicotinic acid	2	4650	193	9,130	85
10% Lard + 1.0% nicotinic acid	2	5550	231	7,650	72
10% Lard + 0.1% nicotinamide	2	5470	228	4,150	39
10% Lard + 1.0% nicotinamide	2	3040	127	7,480	70
10% Lard + 0.1% isonicotinic acid	2	2990	125	4,250	40
10% Lard + 1.0% isonicotinic acid	2	3500	146	4,920	46

[1] Livers from at least two rats (2 to 5) were used per incubation.

TABLE 3

Incorporation of acetate-1-C^{14} into sterols and fatty acids by liver slices from chicks fed nicotinic acid or analogues for 4 weeks

Diet	Incubations[1]	Incorporation			
		Sterol		Fatty acids	
		Total counts	%	Total counts	%
	no.	*c/min*		*c/min*	
4% Soybean oil	12	592	100	73,400	100
4% Soybean oil + 1% nicotinic acid	14	1010	170	53,300	73
4% Soybean oil + 1% nicotinamide	13	1580	267	33,700	45
4% Soybean oil + 1% isonicotinic acid	4	1520	257	39,600	54
4% Soybean oil + 1% benzoic	12	1620	274	43,000	59
4% Soybean oil	4	505	100	59,400	100
10% Lard	4	1180	234	31,700	53
10% Lard + ½% cholesterol	4	78	15.4	55,000	93

[1] At least one chick per incubation.

and INA lowered fatty acid synthesis by 27 to 55%, and increased sterol synthesis by 70 to 174%. The compound which decreased fatty acid synthesis the least (NAc) also caused the lowest increase in sterol synthesis.

When the 4% of soybean oil in the basal diet was replaced with 10% of lard (partially at the expense of sucrose), a marked decrease was noted in fatty acid synthesis (table 3, line 7 vs 6 in agreement with the decreased rate of lipogenesis observed by Hill et al. ('58) when dietary fat was increased. The decrease in fatty acid synthesis was accompanied by a substantial increase in sterol synthesis, despite the fact that the sterol in the lard would be expected to exert the opposite effect (line 8). The addition of 0.5% of cholesterol to the lard diet depressed sterol synthesis to a very low level in agreement with the observations of Tomkins et al. ('53). Thus the present results suggest that a control mechanism exists for the partitioning of acetate between the biosynthesis of fatty acids and sterols and that the balance between these reactions can be altered by relatively simple dietary means. The data of Brady and Gurin ('50), Hotta and Chaikoff ('52), Masoro et al. ('54) and Tepperman and Tepperman ('58) also suggest such alterations under the influence of different physiological stresses.

Effects of niacin and related compounds in vitro. In an extensive series of experiments, slices of normal rat liver were incubated with labeled acetate in the presence of various concentrations of NAc, NAm, INA, DPN or TPN, the molarities ranging from 0.01 to 0.25 for the simpler compounds, and from 0.0001 to 0.01 for the coenzymes. The specific activities of the sterol digitonides from incubations treated with NAc or related compounds were expressed as a percentage of their respective controls and the averages plotted in figure 1, the detailed results being recorded elsewhere.[8] Considerable daily variation was observed in the activities of the sterols from the unsupplemented liver slices, possibly due to variations in the crude diet on which the rats had been maintained. Nevertheless, the responses to NAc *in vitro* were fairly consistent when expressed as "per cent specific activity:"

$$\frac{\text{Sp. act. of sterol of treated sample}}{\text{Sp. act. of sterol of control sample}} \times 100.$$

[8] Hardy, R. W. F. 1960 Ph.D. thesis, University of Wisconsin, Madison.

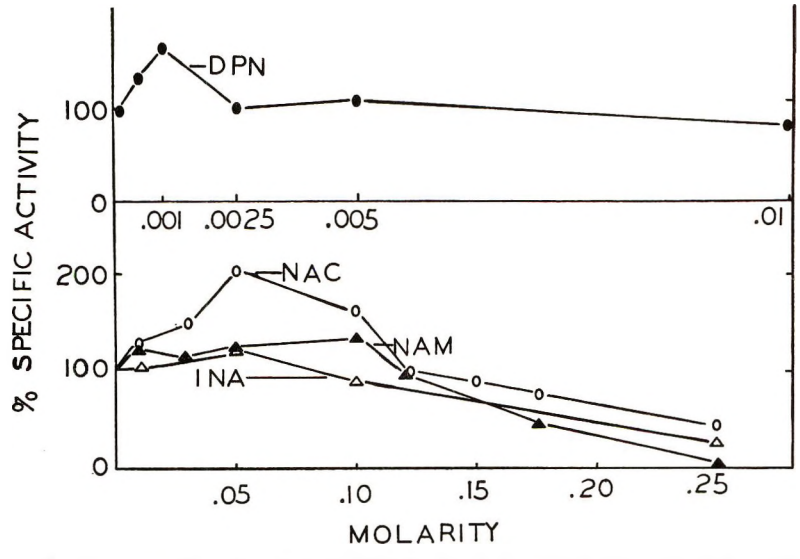

Fig. 1 The incorporation of acetate-1-C^{14} into sterols by incubation of rat liver slices made 0.01 to 0.25 M in nicotinic acid, nicotinamide, or isonicotinic acid, or 0.0001 to 0.01 M in DPN expressed as "per cent specific activities" (text). NAc, NAm, INA and DPN curves represent results of 86, 62, 30 and 40 respective individual samples.

TABLE 4

The incorporation of acetate-1-C^{14} into sterols and fatty acids by rat liver slices exposed to nicotinic acid, nicotinamide and isonicotinic acid in vitro

Compound	Molarity	Sterol		Fatty acid	
		Specific activity	Per cent of control	Specific activity	Per cent of control
		c/min/mg		*c/min/mg*	
Nicotinic acid	0	3458[1]	100	2320	100
	0.0001	3760	108	2090	90
	0.01	3705	107	1350	58
	0.05	5166	149	915	39
	0.10	3175	92	285	12
Isonicotinic acid	0	3458	100	2320	100
	0.0001	2810	82	1960	84
	0.01	3100	90	615	27
	0.05	4180	118	157	5
	0.10	3170	92	21	0.1
Nicotinamide	0	2675	100	937	100
	0.0001	2060	78	724	78
	0.01	2455	92	769	82
	0.05	3280	123	203	22
	0.10	2160	81	152	16

[1] Each value represents the average of duplicate incubations.

In 13 of 19 trials with 0.01 to 1.0 M NAc the per cent specific activity of the sterol was increased by more than 33%, 5 of the remainder showing lesser increases. The most effective concentration of NAc appeared to be 0.05 to 0.1 M. Higher doses of NAc depressed sterol synthesis. NAm or INA were consistently less effective in increasing sterol synthesis than NAc (fig. 1), whereas at high doses, they depressed sterol synthesis like NAc.

Determinations of total oxidized pyridine nucleotides in the incubation mixtures at intervals up to 3 hours showed 0.001 to 0.10 M NAm to retard the destruction of total coenzyme, whereas destruction in the presence of 0.05 M NAc was very similar to that in the control flasks.[9] Thus increased sterol synthesis did not appear to depend upon the total oxidized pyridine nucleotide content of the liver slices.

The addition of DPN to the incubation mixture resulted in increased sterol synthesis at 0.0005 to 0.001 M (fig. 1). Higher concentrations were without effect or they depressed synthesis slightly. Thus the effects of DPN were qualitatively like those with NAc, although quantitatively less marked. An exploratory incubation with TPN indicated it to be less effective than DPN.

The addition of NAc, NAm or INA to liver slices *in vitro* resulted in substantial and progressive decreases in fatty acid synthesis (table 4). The effects on sterol synthesis were less regular, although there were indications of increased sterol synthesis in the presence of 0.05 M NAc or its analogues. Concentrations of 0.05 M NAc, NAm or INA depressed fatty acid synthesis after only 30 minutes of incubation (fig. 2) at which time the rate of sterol synthesis was unaltered, whereas at 90 minutes the effects of the compounds upon sterol synthesis were evident. The greater and more rapid effects of the compounds upon the incorporation of acetate into fatty acids suggest that this is the process primarily affected by the niacin analogues, and that the observed changes in sterol synthesis represent secondary effects incidental to the primary change.

DISCUSSION

In these experiments appropriate levels of acetate were incorporated into fatty acids and sterols at rates which varied with the presence of nicotinic acid or related substances. Several lines of evidence suggest that the primary effect of NAc or its analogeus was to decrease fatty acid synthesis and that the observed increases

[9] See footnote 8.

in sterol synthesis were probably secondary effects. The decreases in fatty acid synthesis were never appreciably less than the accompanying increases observed in sterol synthesis (tables 2, 3, 4) and often the changes in fatty acids were substantially greater; depressions in fatty acid synthesis occurred at lower levels of added NAc or analogues than the elevations in sterol synthesis (table 4); and a marked depresison in fatty acid synthesis due to NAc or its analogues occurred after only 30 minutes of incubation when no elevation of sterol synthesis was yet apparent (fig. 2). It would therefore appear that the observed increase in sterol synthesis represented one route for the disposal of acetate that was not incorporated into fatty acids.

Others have reported that an altered partitioning of acetate between sterol and fatty acids by liver slices can occur under a variety of circumstances. A simultaneous depression of fatty acid synthesis and an elevation of sterol synthesis has been found in alloxan diabetes (Hotta and Chaikoff, '52, Brady and Gurin, '50), and in livers of rats exposed to a cold environment (Masoro et al., '54). Conversely in a re-feeding experiment Tepperman and

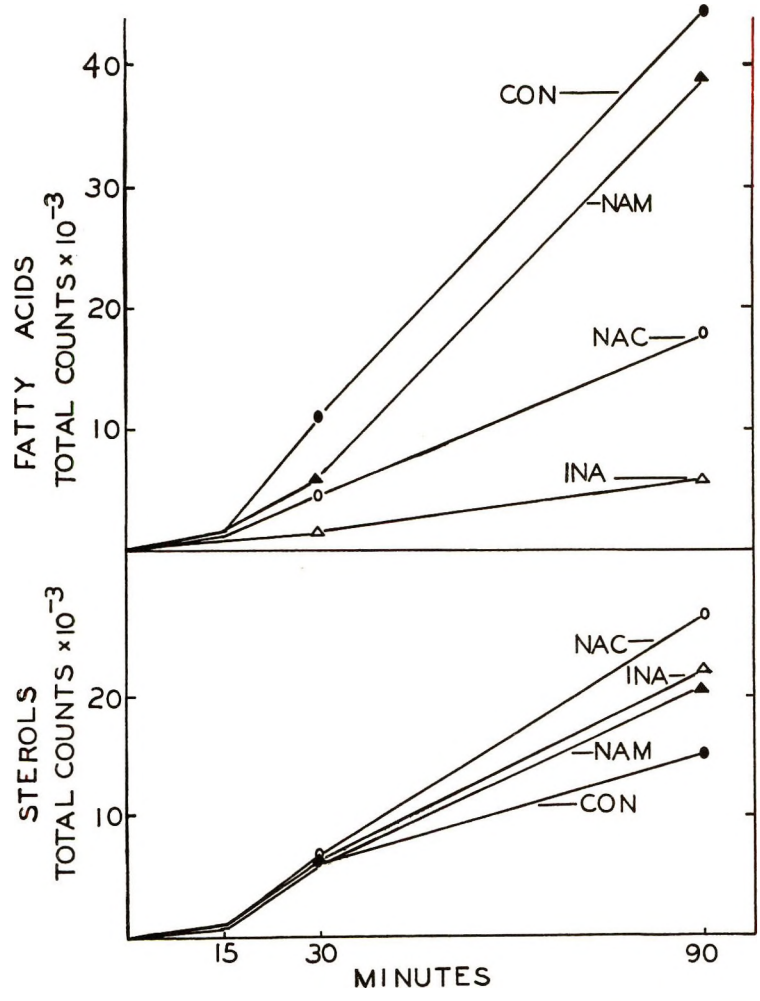

Fig. 2 Incorporation of acetate-1-C^{14} into fatty acids and sterols after various times of incubation by rat liver slices treated *in vitro* with 0.05 M nicotinic acid, nicotinamide or isonicotinic acid.

Tepperman ('58) observed an increase in lipogenesis and a decrease in sterolgenesis. It has been suggested that these latter alterations in fatty acid synthesis may be due to changes in the amount of TPNH generated from carbohydrate glycolysis by way of the hexose monophosphate shunt (Shaw et al., '57, Tepperman and Tepperman, '58). An altered partitioning of acetate between sterols and fatty acids does not occur in the disorganized cell: Siperstein and Fagan ('58) failed to observe an elevation in sterol synthesis in liver homogenates from diabetic rats, although fatty acid synthesis was depressed.

In our previous study, NAc or INA lowered the level of blood cholesterol in the chick, and INA lowered it in the rat (Gaylor et al., '60). The present results show that this lowering cannot be attributed to any impairment of hepatic sterol synthesis since liver slices from rats or chicks fed these compounds actually showed an increased ability to incorporate acetate into sterol. In all probability the blood cholesterol-lowering effect of INA or NAc involves at least a bimodal response, i.e., enhanced sterol synthesis accompanied by an increase in mechanisms which remove cholesterol from the blood. In the rat which normally has a low level of blood cholesterol NAc appears to cause the former effect to predominate, whereas in the chick which normally has a higher level of blood cholesterol, the latter response appears to predominate.

SUMMARY

1. The incorporation of acetate-1-C^{14} into sterols and fatty acids was examined with liver slices from rats and chicks fed nicotinic acid (NAc), nicotinamide (NAm), isonicotinic acid (INA) or benzoic acid; these compounds were also added to liver slices *in vitro*.

2. Liver from rats or chicks fed NAc or related compounds showed an increased incorporation of acetate into sterols and a decreased incorporation into fatty acids. This altered incorporation varied with the length of time during which NAc or related compounds had been fed.

3. Incubations of rat liver slices in a buffer 0.01 to 0.10 M in NAc, NAm, or INA resulted in substantial decreases in fatty acid synthesis, the most marked depressions occurring with INA; this was accompanied at certain concentrations by increases in sterol synthesis, the more marked elevations occurring with NAc. Significant depressions in fatty acid synthesis with 0.05 M NAc, NAm or INA occurred before changes in sterol synthesis were detectable.

4. These results, plus observations by others, suggest that a primary change in fatty acid synthesis may determine an altered partition of acetate between fatty acids and sterols.

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Intestinal Flora of the Pig as Influenced by Diet and Age[1]

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The importance of the intestinal flora of the monogastric animal has been analyzed by Johansson and Sarles ('49) and Mickelsen ('56). Their citations reveal a multitude of reports which in most instances show little agreement with regard to numerical relationships of bacterial groups. For example, in studies using the pig, Bridges et al. ('52, '53), Horvath et al. ('58) and Larson and Hill ('55) all reported coliforms to be predominating. Conversely, Quinn et al. ('53b) as well as Willingale and Briggs ('55), found lactobacilli to be the most numerous fecal microorganisms. Because of such discrepancies the studies reported herein were conducted to clarify the influence of diet, age, preexperimental environment and management on intestinal microorganisms in the pig.

EXPERIMENTAL

All pigs were farrowed and reared to two weeks of age in farrowing stalls on concrete floors and had access to the sow's feed, water and feces during the two-week nursing period. Each pig received 2 ml of an iron-dextran complex (50 mg Fe/ml) intraperitoneally on the first day of age to prevent anemia. The male pigs were castrated at three to 4 days of age, and all pigs were given a mixture of hog cholera and erysipelas antiserum when 5 days old. At two weeks of age the pigs were removed from their dams and placed in an isolated building equipped with radiant floor heating (individual pens only), air conditioning and ultraviolet germicidal lamps. The pigs received feed and water ad libitum, and the pens were cleaned daily, with no bedding being used.

Two experiments of randomized block design were conducted with 24 pigs each. The pigs were randomly allotted from weight outcome groups within litters to one of two semipurified diets containing either raw corn starch or lactose as the source of carbohydrate (table 1). One-half of the pigs were penned individually and the other half in groups of 6 (all within the same building). The experimental period was 28 days (14 to 42 days of age), and half of the pigs were killed when 42 days

TABLE 1
Composition of the diet

Ingredient	%
Casein (acid ppt.)	22.73
Corn starch (or lactose)	67.70
Cellulose[1]	2.00
Stabilized lard	2.00
Monocalcium phosphate	2.29
Calcium carbonate (ca. 38%)	0.43
Iodized salt	0.50
Trace mineral premix[2]	2.00
Vitamin premix[3]	0.30
Saccharin	0.05

[1] Wood-Floc, Brown Company, Chicago.

[2] Supplied the following elements in parts per million: Fe, 362; Cu, 7.6; Co, 3.1; Zn, 50; Mn, 101.5; K, 3980; I, 0.4; Mg, 530.

[3] Supplied the following per kilogram of diet: α-tocopheryl acetate, 22.0 mg; ascorbic acid, 220.5 mg; biotin, 6.61 μg; Ca pantothenate, 21.96 mg; folic acid, 1.10 mg; inositol, 6.55 gm; menadione, 1.10 mg; niacin, 65.92 mg; *p*-aminobenzoic acid, 4.41 mg; pyridoxine·HCl, 3.31 mg; riboflavin, 9.26 mg; thiamine·HCl, 6.61 mg; vitamin A, 6610 I.U.; vitamin B_{12}, 44.1 μg; vitamin D_2, 2205 I.U.

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old. Microbial counts were made on the contents of the duodenum, the distal 100 cm of the ileum, the cecum and the rectum. All data were subjected to analysis of variance as outlined by Snedecor ('56).

Pig weight and feed consumption were determined each week, at which time rectal samples were taken. All animals were observed at least twice daily for any abnormal symptoms, as well as for consistency of the feces, and at no time during the experiments were any chemotherapeutics administered orally or otherwise. Animal sacrifice was carried out by passing a 110 volt current through the pig for approximately 15 seconds, and samples were taken by opening the abdominal cavity and tying off the various sections of the intestinal tract to be studied. These sections were then removed and the contents extruded into sterile containers. The entire process took no more than 10 minutes per pig, and the samples were immediately taken to the laboratory for assay. Animals were killed or rectal samples were taken at approximately the same time of day in all experiments (8 to 9 A.M.).

Each sample was handled individually and the range of sample size was one to 10 gm. Standard plate count techniques with selective media were used for all organisms studied. The samples were diluted, initially by weight, one part of sample to 9 parts of sterile buffered water. This mixture was then blended for two minutes in a semimicro Waring blendor and serial decimal dilutions were made in buffered water. Each of three plates was inoculated with 1 ml of the appropriate dilution, and as each plate was inoculated 18 ml of the required medium were added by pipetting machine.

The groups of organisms studied were: the total aerobes (tryptone glucose extract agar), total anaerobes (thioglycollate medium plus 1.5% of agar), lactobacilli (tomato juice agar, special) coliforms (violet red bile agar), streptococci (mitis salivarius agar plus potassium tellurite), staphylococci (staphylococcus medium no. 110), and molds and yeasts (Littman oxgall agar plus 30 μg/ml of streptomycin and 20 units/ml of penicillin). The media were sterilized by autoclaving for 15 minutes at 121°C. The antibiotic and tellurite solutions were added aseptically after sterilization.

The thioglycollate plates were placed in vacuum desiccators, and the atmosphere was removed with a water aspirator-type vacuum pump and replaced with natural gas. The Littman oxgall plates were incubated at 30°C for three to 5 days (depending on the rate of mycelial growth), the violet red bile plates at 37°C for 18 to 24 hours, and the others at 37° for 48 hours. The composition of the selective media and details for their use have been published elsewhere.[5]

RESULTS

Animal performance. General condition and performance of the animals in this study were considered to be good, with the average initial weight at two weeks of age being 4.84 kg and the average final weight at 6 weeks of age, 10.61 kg. Both 4-week gain and feed required per unit of gain were found to be far greater in those pigs fed diets containing lactose in comparison with those fed starch (gain: 7.17 vs 4.35 kg and feed/gain: 1.45 vs 1.96). Also highly significant differences in 4-week gains were found between group- and individually-fed pigs, the average gains being 5.31 and 6.26 kg, respectively. Feed utilization by the group-fed pigs was superior (1.62 vs. 1.92); however, this was considered to be a result of less feed wastage due to mechanical differences in the feeders.

Rectal bacteria (freshly voided feces). The data demonstrating diet and time effects are graphically summarized in figure 1. The results for over-all diet and management effects are summarized in table 2. Lactose, as compared with starch, produced a highly significant reduction in the number of all rectal organisms studied, with the exception of the total anaerobes and lactobacilli. Significant differences in the total aerobes, total anaerobes, staphylococci and molds and yeasts were also found between litters. Statistical analysis also demonstrated a significant effect of time on population densities of all organ-

[5] Difco Laboratories, Inc. 1953 Difco manual of dehydrated culture media and reagents for microbiological and clinical laboratory procedures, ed. 9. Detroit.

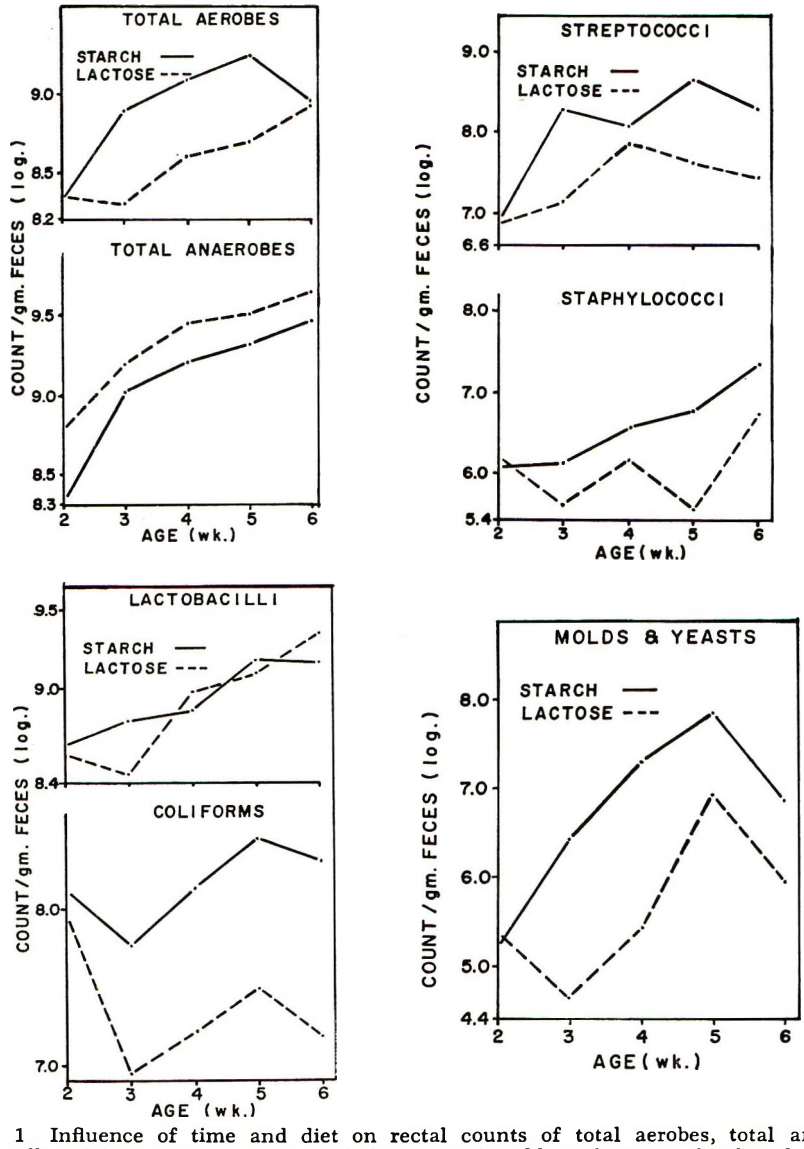

Fig. 1 Influence of time and diet on rectal counts of total aerobes, total anaerobes, lactobacilli, coliforms, streptococci, staphylococci and molds and yeasts. Combined summary of the individually- and group-fed pigs.

isms studied, as well as interactions of diet and time for the standard plate counts of total aerobes, streptococci, staphylococci and molds and yeasts.

With regard to management, individually-fed pigs generally had lower counts than group-fed pigs for all organisms studied, but statistically significant differences were noted only for the total aerobes, total anaerobes and lactobacilli. Thus, diet as well as time and diet interactions had predominant effects upon the total aerobes, coliforms, streptococci, staphylococci and molds and yeasts; but management was apparently the controlling influence on the total anaerobes and lactobacilli. Possibly the dietary effects were great enough on the majority of organisms to mask any effects of management.

Intestinal bacteria. The data for effects of diet on the flora within various sections of the intestinal tract are presented in fig-

TABLE 2

Average rectal counts of all organisms for management and diet effects[1]

Organism	Diet	Management	
		Group-fed	Individually-fed
Total aerobes	starch	9.01	8.74
	lactose	8.66	8.40
Total anaerobes	starch	9.22	9.03
	lactose	9.36	9.15
Lactobacilli	starch	9.06	8.75
	lactose	8.94	8.68
Coliforms	starch	8.40	7.87
	lactose	7.38	7.45
Streptococci	starch	8.08	7.92
	lactose	7.50	7.15
Staphylococci	starch	6.46	6.49
	lactose	6.16	5.75
Molds and yeasts	starch	6.73	6.53
	lactose	5.67	5.58

[1] All counts are given as the log per gram of feces (wet).

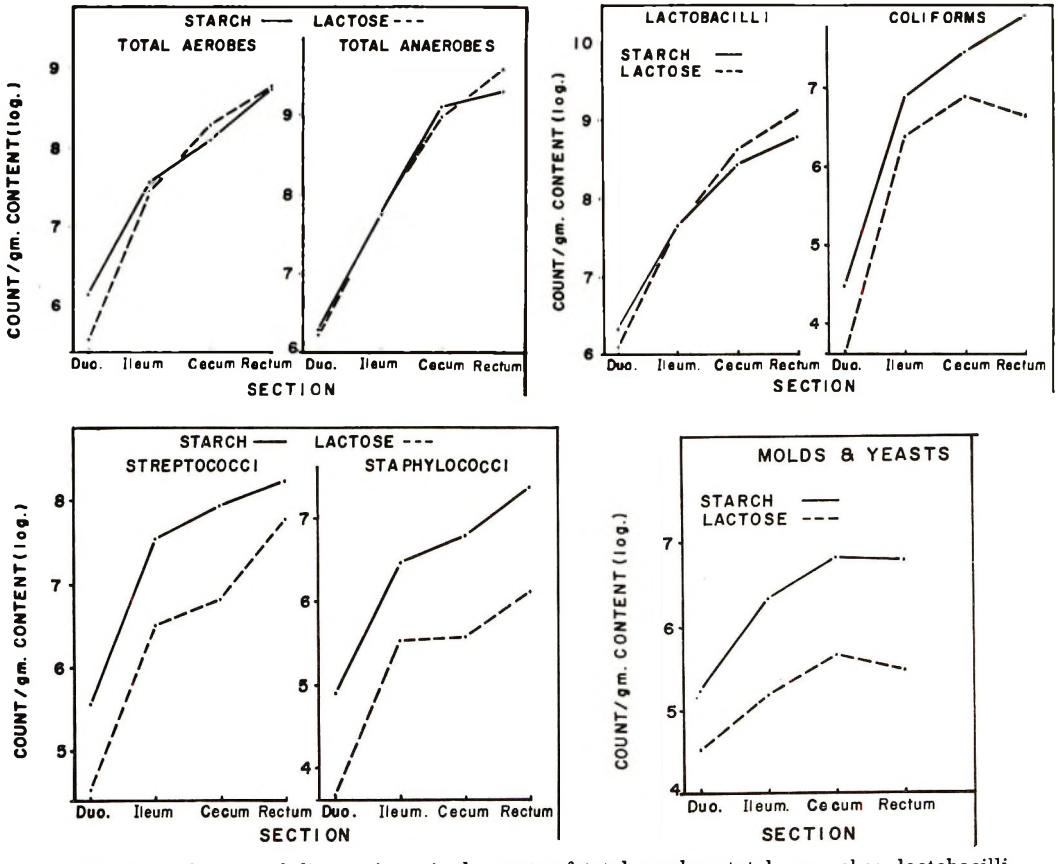

Fig. 2 Influence of diet on intestinal counts of total aerobes, total anaerobes, lactobacilli, coliforms, streptococci, staphylococci and molds and yeasts. Combined summary of the individually- and group-fed pigs.

TABLE 3
Average pH values of various sections of the intestinal tract

Section	Diet	
	Starch	Lactose
	pH	*pH*
Stomach	4.1	3.7
Dudoenum	5.4	5.1
Ileum	6.3	6.8
Cecum	6.1	5.6
Rectum	7.0	6.8

ure 2. Again, lowered counts were produced by dietary lactose. The differences throughout the digestive tract approached significance for the coliforms and were highly significant for the streptococci, staphylococci and molds and yeasts. Litter effects were found to be quite strong for all groups studied, with the exception of the coliforms. The only groups that management, individually-fed vs group-fed, significantly influenced were the lactobacilli and streptococci in which the group-fed pigs had higher counts than the individually-fed pigs. As would be expected, the counts of all organisms differed significantly between sections of the intestinal tract under each management regimen.

Table 3 presents a brief summary of the average pH values for the various sections of the intestinal tract with respect to the influence of diet. With the exception of the ileum, lactose feeding resulted in a lower pH than did starch in each section measured.

General observations. Data not presented here, on samples taken at periodical intervals in the growth of the pig from birth to market weight, indicate that the intestinal tract of the newborn pig is relatively devoid of microbial growth, but within 24 hours these populations reach their greatest density in terms of viable cells per gram of contents. For example, the total aerobes, total anaerobes, and lactobacilli all approached log counts of 10.0 per gm of fresh content (rectal) at one day of age and then slowly declined to about 8.5 at two weeks of age, at which time all pigs in these studies were weaned. Following weaning to dry diets, these counts rose to just below the level they had attained at one day of age and then remained fairly stable with a declining tendency up to market weight. Similar patterns were also observed for the streptococci, staphylococci and molds and yeasts but at lower counts. The coliform organisms, however, showed an ever decreasing count from one day of age to market weight, with only a brief increase between three and 5 weeks of age.

DISCUSSION

The physiological effects of feeding lactose to various animals has recently been reviewed (Atkinson, '57), and it has been demonstrated that, unlike other animals studied, the young pig and dairy calf can tolerate very high levels of lactose in the diet, which agrees with the findings presented here.

The more rapid gains of the individually-fed pigs might be related to less competition between pigs as compared with group feeding, even though adequate feeder space was provided for the latter. It is also possible that the intestinal flora plays an important role as the counts of almost all organisms were lower in the individually-fed pigs. This has also been noted by Willingale and Briggs ('55) with older pigs. Furthermore, the generally lowered coefficients of variation with group feeding (table 4) indicate that there is definitely some influence of one pig on another with respect to intestinal microorganisms.

The sharp rise in count immediately following weaning might appear to be the result of a concentrating effect brought about by rather low feed consumption during this period (table 1). However, closer observation reveals that the majority of organisms from the lactose-fed pigs changed only slightly in population density during the first week, reflecting the similarity of pre- and post-weaning diets; conversely, the starch diet provided a definite change in substrate for the intestinal flora. The increase in molds and yeasts and the concomitant reduction in coliform count has also been previously observed by Quinn et al. ('53b).

The general tendency for increasing counts over most of the experimental period is more difficult to understand. However, the recent findings on digestive enzymes of the baby pig (Bailey et al., '56; Hudman et al., '57; Lewis et al., '57) could

partially explain the effects observed. For example, the rather poor utilization of starch in the initial period provided relatively large quantities of this undigested carbohydrate as substrate in the large intestine, and the pig's ability to utilize the starch increases rapidly with age. Conversely, the pig's ability to utilize lactose is very high initially, and is generally considered to decrease with increasing age. This would provide more available carbohydrate for microbial growth in the posterior area of the digestive tract as time elapses, yet not to the extent realized by starch feeding. The lactose may also be exerting some of its effect through an increased rate of food passage. With organisms which do not respond to the above patterns of carbohydrate availability, other factors, such as variable protein utilization, pH levels and gastrointestinal secretions, may be more intimately involved.

The counts obtained from the sacrificed animals follow a pattern of increasing numbers from the anterior to posterior ends of the intestinal tract, which is generally considered to be typical for most species. The differences observed cannot be related directly to pH alone, as careful examination of these comparisons revealed some inconsistencies. Similarly, the differences in dilution cannot be the greatest determining factor as cecal counts were closer to rectal (fecal) than to ilial counts, but the percentage of moisture for cecal contents was closer to that found in the ilial contents. There were no apparent differences in moisture with different types of carbohydrate. Some of the more important controlling factors may be related to intestinal secretions and to a differential rate of turnover throughout the various sections. This turnover is probably greater in the duodenum and ileum than in the cecum and rectum.

The insignificant differences between diets with respect to their effects on counts of total aerobes, total anaerobes, and lactobacilli throughout the intestinal tract are in agreement with results of Larson and Hill ('55) and of Willingale and Briggs ('55) who suggest that these populations are fairly stable. Dietary influences on the coliforms, streptococci, staphylococci and molds and yeasts observed in these studies are contradictory to those of the above

TABLE 4

Coefficients of variation (as percentage)[1]

Organism	Source[2]	Management	
		Group	Individual
Total aerobes	E-1	3.7	6.0
	E-2	4.8	5.0
	S	4.9	10.1
Total anaerobes	E-1	3.6	6.2
	E-2	6.5	6.3
	S	8.2	8.4
Lactobacilli	E-1	4.3	5.7
	E-2	4.5	6.5
	S	5.3	8.4
Coliforms	E-1	9.1	11.4
	E-2	8.0	9.8
	S	12.1	11.5
Streptococci	E-1	9.4	9.3
	E-2	5.7	8.7
	S	6.7	10.4
Staphylococci	E-1	7.4	12.4
	E-2	6.9	14.7
	S	10.2	16.6
Molds and yeasts	E-1	9.8	15.3
	E-2	7.9	9.2
	S	14.7	11.3

[1] $s/\bar{x} \times 100$.

[2] E, experiment; S, sacrifice data.

workers as well as Horvath et al. ('58), who concluded that the intestinal flora was "buffered" and not readily subject to change. Conversely, Quinn et al. ('53a) concluded from their work that there was no "normal flora" in the pig and suggested that the existing flora was a reflection of the quality and quantity of ration consumed.

All of these workers used practical-type diets as well as different management procedures, which would provide numerous sources of variation, making it much more difficult to repeat accurately work of this nature and to compare directly results obtained at different times and places. For example, Bridges et al. ('52, '53) reported that the coliforms, staphylococci and species of *Proteus* and *Shigella* predominated in pigs 7 to 16 weeks of age. Horvath et al. ('58) similarly found the coliforms to be numerically predominant, with the lactobacilli and enterococci somewhat lower in numbers. Throughout their experiments, Larson and Hill ('55) found the coliforms to be relatively high in numbers; but the lactobacilli varied from very low counts to being the predominating organisms.

The results reported herein are in general agreement with those observed by Quinn et al. ('53b) as well as Willingale and Briggs ('55), in that the lactobacilli were the numerically predominating organisms followed by a comparatively lower coliform count. Furthermore, the rather high counts obtained for molds and yeasts agree with those of Quinn et al. ('53a) as compared with lower values observed by other workers (Horvath et al., '58; Larson and Hill, '55).

From these and other studies it can be seen that the major problem which arises in considering the intestinal or fecal microorganisms is that of variation, which is not necessarily simply of animal origin, but may actually be largely the result of inconsistencies in experimental techniques. An example of animal variation, however, can be found in the significant influence of replication and litter on various bacteriological counts demonstrated in these studies. Although a certain portion of replication effects can be ascribed to laboratory variation, litter effects must be directly related to physiological characteristics of the individual pig as influenced by genetics and the environment immediately preceding the experimental period. The interaction of these effects with subsequent treatments imposed on the animals further amplifies the need for careful planning and controlling of experimental variables in studies of this nature.

SUMMARY

Studies have been conducted on the intestinal and rectal microorganisms of the pig to determine how they vary with diet, age, pre-experimental environment and management. The influences of diet and management were studied using 48 pigs weaned at two weeks of age and fed to 6 weeks of age, at which time half of the pigs were sacrificed and bacteriological examinations made on duodenal, ilial, cecal and rectal contents. The organisms studied were the total aerobes, total anaerobes, lactobacilli, coliforms, streptococci, staphylococci and molds and yeasts.

With respect to gain and efficiency of feed utilization, lactose was far superior to raw corn starch, and individual feeding resulted in faster gains than group feeding. All rectal organisms, with the exception of the total anaerobes and lactobacilli, were lower in numbers when lactose, as compared with starch, was the carbohydrate fed. In each section of the intestinal tract sampled, the coliforms, streptococci, staphylococcus and molds and yeasts were also lower in numbers with lactose feeding. Counts of most organisms increased sharply from the duodenum to cecum and only slightly from the cecum to rectum.

Pigs fed in groups of 6 generally had higher counts with less variation than those individually-fed. Also, a significant difference between litters was observed.

The intestinal tract of the newborn pig is relatively devoid of microbial growth, but within 24 hours these populations reached their greatest density in terms of viable cells. Most of the organisms studied showed a general decline in numbers from one day of age up to weaning (2 weeks of age), at which time they increased in numbers to a level just below that attained at one day of age. The counts then remained fairly stable to market weight.

The coliforms, however, seem to follow a general tendency to decline in numbers from one day of age to market weight.

Of the groups of organisms studied, the lactobacilli generally predominated in numbers, with the streptococci, coliforms, staphylococci and molds and yeasts usually following in that order. These relationships were influenced to a certain extent by age and also by location in specific sections of the intestinal tract.

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Amino Acid Requirements of Children

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The quantitative amino acid requirements have been studied using infants (Holt, '59), adult males (Rose et al., '55) and females (Leverton et al., '56). However, no studies of school children have been made. Besides, DL-amino acids have been often used in the above experiments, but the unphysiological effects of D-amino acid must be taken into consideration. Several patterns for amino acid mixtures have been designed for determining amino acid requirements. Leverton et al. ('56) fed the essential amino acids daily in the same quantitative ratio as they occur in 20 gm of egg protein. Pratt et al. ('55) fed infants amino acid mixtures equivalent to those in breast milk. Since no pattern had been suggested for school children, we used that set by Rose et al. ('55) for the benefit of comparing the requirements of children with those of adults, and the minimal required amounts of all 8 essential amino acids were fed. However, we added the same amount of histidine as tryptophan in our pattern. Histidine has been regarded as unessential for adults, but, in the studies of Holt ('59) on infants it appeared to be essential. Therefore we regarded it tentatively as essential for children.

SUBJECTS AND METHODS

Experiment 1. Three boys, 13 to 14 years old served as subjects. They lived in an institute under our supervision. The boys were weighed daily before breakfast throughout the experimental period of 10

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TABLE 1
Composition and daily intake of experimental diets

Component	Diet 1[1]		Diet 2[2]	
	Daily intake	Approximate calories	Daily intake	Approximate calories
	gm		*gm*	
Cornstarch	180	720	300	1200
Sucrose[3]	200	800	200	800
Butter fat	30	270	60	540
Corn oil	30	270	50	450
Salt mixture[4]	4.7		4.7	
Vitamin mixture[5]	1.0		1.0	
Baking powder	5.4		7.0	
Sodium chloride	5.8		11.0	
Cellulose powder (carboxymethyl cellulose)	10		18	
Agar	1.5		2.0	
Drops[6]	110	440		
Total		2500		2990

[1] For children.
[2] For adults.
[3] A larger amount of sugar was used to improve the palatability of amino acid mixture.
[4] In grams, $CaCO_3$, 1.510; $KHCO_3$, 2.360; $MgSO_4 \cdot 7H_2O$, 0.648; $FeC_6H_5O_7 \cdot 6H_2O$, 0.175; and in milligrams, $MnSO_4 \cdot 4H_2O$, 2.22; $CuSO_4 \cdot 5H_2O$, 7.85; KI, 0.442 (Rose et al., '50).
[5] Thiamine, 5.0 mg; riboflavin, 1.5 mg; vitamin B_6, 0.5 mg; vitamin B_{12}, 1.0 μg; niacin, 10.0 mg; folic acid, 0.5 mg; D-Ca-pantothenate, 0.5 mg; vitamin C, 37.5 mg; vitamin K_4 diacetate, 0.2 mg; vitamin A palmitate, 2500 I.U.; vitamin D_2, 200 I. U.; vitamin E, 1.0 mg.
[6] Candy made of sugar only.

to 14 days. The basal diet consisted of the materials listed in table 1, which were baked as wafers and biscuits, or cooked as puddings and doughnuts.

The composition and the daily intake of essential amino acid mixtures used as a base line are shown in table 2.

TABLE 2

Composition and daily intake of essential amino acid mixture

Component	Mixture A[1]	
	Daily intake	Nitrogen content
	gm	*gm*
L-Valine	0.80	0.096
L-Leucine	1.10	0.118
L-Isoleucine	0.70	0.075
L-Methionine	1.10	0.103
L-Phenylalanine	1.10	0.093
L-Threonine	0.50	0.059
L-Lysine	0.80	0.153
L-Tryptophan	0.25	0.034
L-Histidine	0.25	0.068
Total		0.799

[1] Minimal requirements by Rose et al. ('55).

Glycine was the other source of nitrogen and made up the difference between the nitrogen supplied by 9 essential amino acids and the total nitrogen intake. One third of these mixtures was weighed for each subject for each meal. Sufficient hot distilled water was used to mix all of the amino acids into a solution, and adequate amounts of sugar were added to improve the palatability of the solution, which was chilled before serving.

The nitrogen balance method, or the intake minus excretion, was used to determine the minimal amino acid requirements of children, and the positive nitrogen balance was regarded as the criterion of adequacy. Daily nitrogen balances were determined during a period of two or three days in order to demonstrate that the subject was capable of maintaining nitrogen balance. The level of essential amino acids or total nitrogen intake was changed in each period. Nitrogen of diet, urine and feces was analyzed by the macroKjeldahl method. An aliquot of the daily diet was homogenized and kept for analysis. Twenty-four-hour urine samples were collected each day. The daily output of feces was homogenized with water, made up to a certain volume with additional water and mixed thoroughly. One tenth of this was kept under refrigeration. At the end of the experimental period, the homogenized material was mixed and an aliquot taken for analysis. The average daily excretion of fecal nitrogen was obtained by dividing the total output by the number of days in the period. Fecal nitrogen values did not vary greatly day by day for the same child. Urinary creatinine was determined by the method of Peters ('42).

Results, experiment 1. The first subject, N. Y., was a healthy boy, 13 years old (fig. 1). He received initially the amino acid mixture A, (table 2), and the total nitrogen with the addition of glycine amounted to 9 gm. He received the diet for two days preceding the collection of excreta in order to allow him to become accustomed to the diet and to living in a laboratory. On the third and 4th days he showed a negative balance with the diet mentioned above. On the 5th day the diet was modified to contain 11 gm of total nitrogen by increasing glycine. However, the nitrogen balance still remained negative. Then the mixture of 9 essential amino acids was doubled in quantity (mixture B) but the total nitrogen intake was kept at a constant level by subtracting isonitrogenous amounts of glycine. He still maintained a negative nitrogen balance, but by increasing glycine, to make a total nitrogen content of 14 gm, he came into positive balance. Following this, the 9 essential amino acids were reduced by half, maintaining a constant total nitrogen level by the substitution of glycine. The subject then promptly came into negative balance again. Throughout the experiment, body weight and the excretion of creatinine remained constant.

The second subject, S. H., (fig. 1) was a boy 14 years old. He came into positive nitrogen balance when receiving twice the amount of the essential amino acid mixture (mixture B) required by adults, with total nitrogen maintained at a level of 14 gm. However, the boy lost 1.5 kg of weight during the experiment, because of poor appetite.

The third boy, aged 14 years, received from the beginning the essential amino acid mixture B at a 14-gm level of total nitrogen, resulting in positive nitrogen bal-

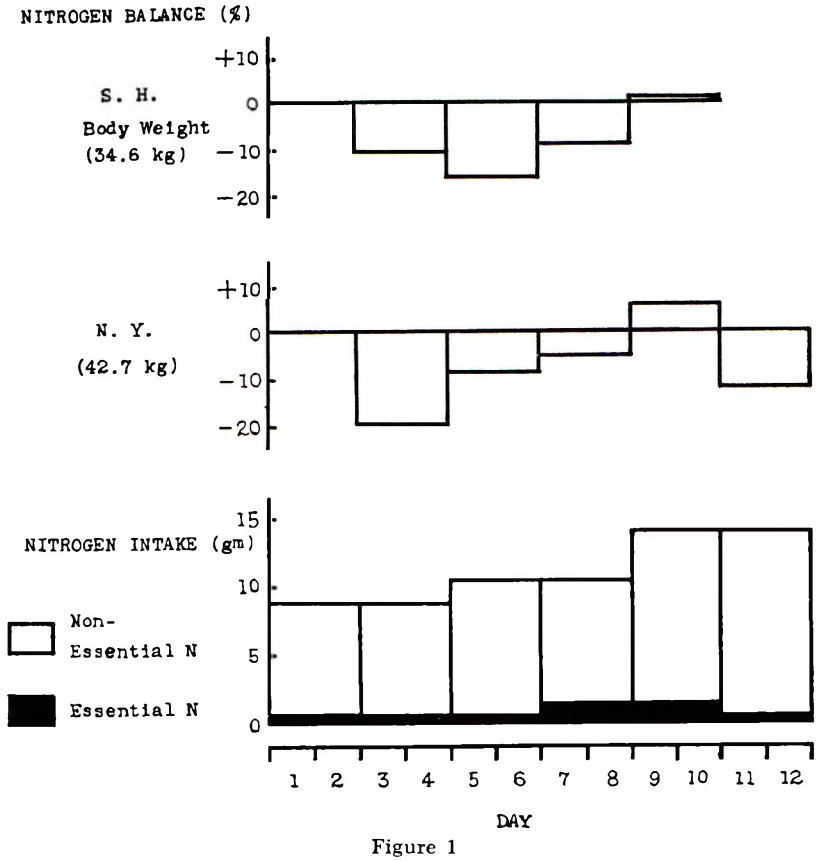

Figure 1

ance. Following this, tryptophan was totally withdrawn from the amino acid mixtures, replaced by isonitrogenous amounts of glycine, and the subject came into negative balance at 115% of the intake.

Experiment 2. In the previous experiment we observed that the requirement of children for essential amino acid mixtures was double the minimal amino acid requirements of adults as determined by Rose et al. ('55).

We then wished to ascertain whether children who weigh half as much as adults require twice the amount of amino acid mixtures. A healthy woman, 21 years old, served as a subject, living in our laboratory and eating a diet composed of the same food materials used in the first experiment.

However, we modified the nonessential amino acid mixtures by substituting a larger part of glycine with L-glutamic acid, L-Na-glutamate and L-arginine, because a larger amount of glycine tended to lessen the appetite. Also, amino acids were administered in the form of pills rather than in solution, and essential amino acid mixtures were swallowed by the use of wafers. Total daily intake of amino acids was divided into three portions and fed at each meal. In this experiment the urine was analyzed not only for total nitrogen and creatinine, but also for creatine.

Results of experiment 2. The subject was active, did not lose her appetite and maintained body weight at a constant level (63 kg) during the 11-day experiment.

After a preliminary period of two days, the excreta were collected and checked for nitrogen balance. The nitrogen equilibrium was maintained with twice the amount of the minimal requirement defined by Rose et al. ('55). The nitrogen equilibrium is defined, according to Leverton et al. ('56), as the zone in which the difference between the intake and excretion does not exceed $\pm 5\%$, i.e., the excre-

tion is within 95 to 105% of the intake.

Next, the subject was given an amount of essential amino acid mixture A equivalent to the minimal requirement determined by Rose et al. ('55), and scarcely maintained the nitrogen equilibrium. Therefore our result seemed to coincide with that of Rose et al. ('55), as far as the adult is concerned. Furthermore, when we decreased the amount of essential amino acid mixture A by half, without changing total nitrogen amounts, the subject came clearly into negative balance. The excretion of the creatine and creatinine stayed almost at a constant level.

Experiment 3. Five healthy boys, aged 12 years, served as subjects for an experiment designed to confirm the results obtained in the first experiment. They lived in the laboratory under our supervision during the 12-day experimental period. Methods used were almost the same as those described above. The nonessential amino acid mixtures were administered as pills and the essential amino acid mixtures were swallowed by the use of wafers. The basal diet, composed of the same materials used in the first and second experiments, was supplemented with mineral and vitamin mixtures.

Results of experiment 3. During the experimental period, the 5 children lived actively and maintained body weight at a constant level. The nitrogen balance method served as the criterion of adequacy of the rations.

The subjects were unable to maintain positive nitrogen balance by the intake of the essential amino acid mixture A, at a 10-gm level of total nitrogen. Therefore, we gave the essential amino acid mixture B, keeping total nitrogen at a 10-gm level by subtracting isonitrogenous amounts of nonessential amino acids (figs. 2–3). With this, the subjects remained in negative balance but to a lesser degree. By increasing

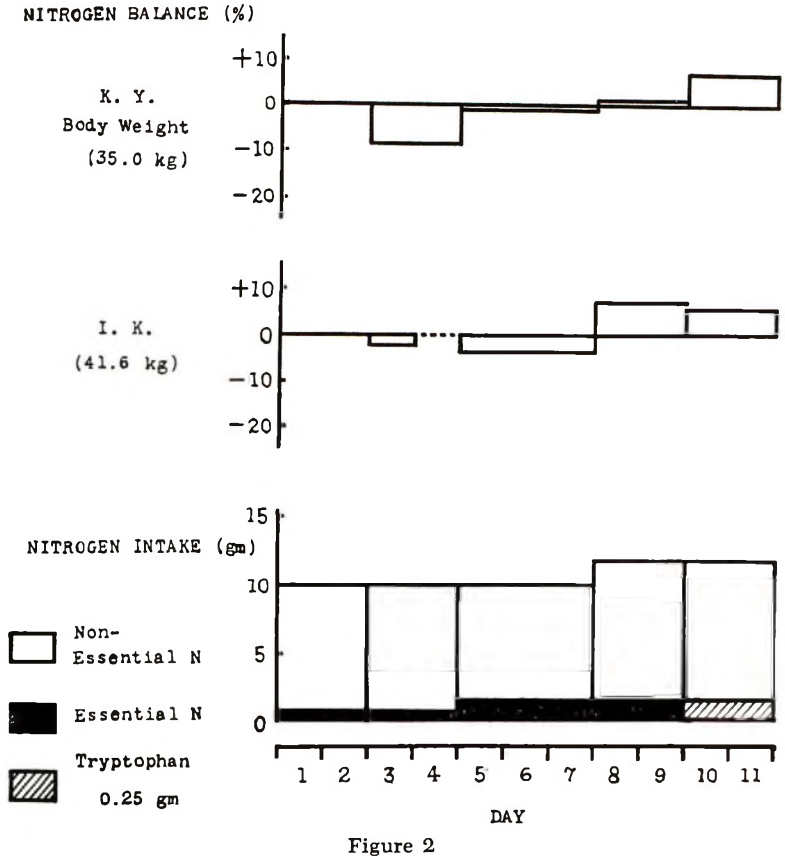

Figure 2

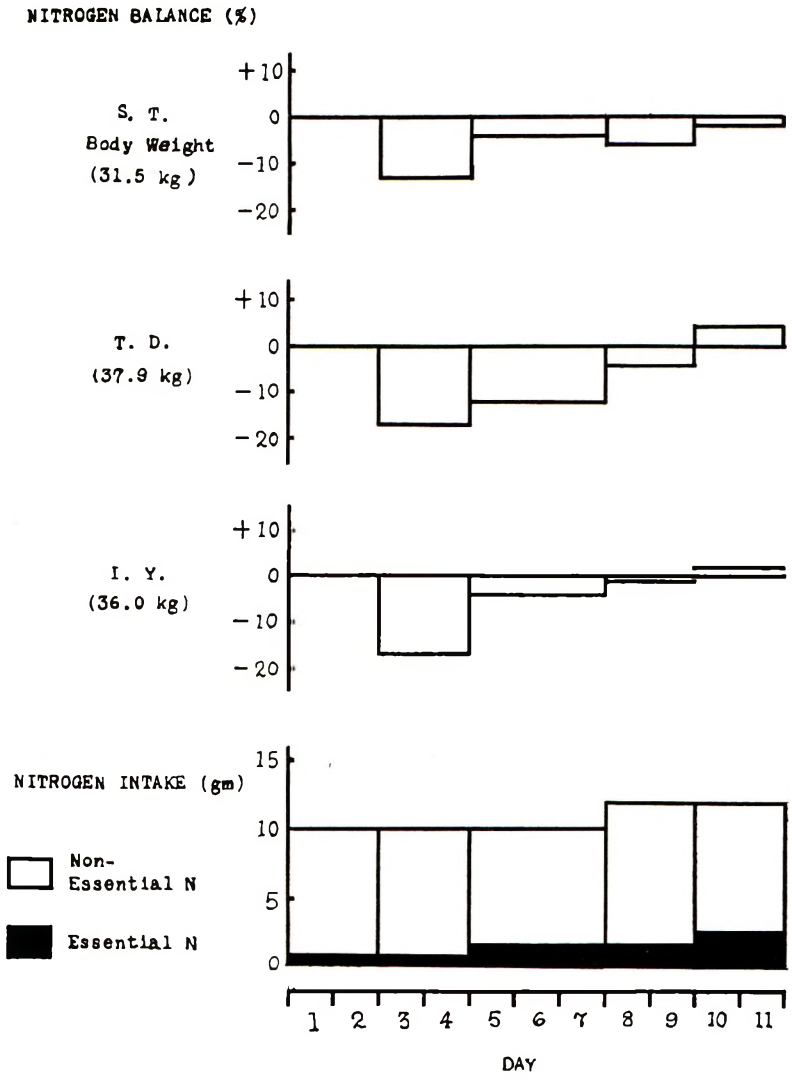

Figure 3

the total nitrogen to 12 gm by the addition of nonessential amino acids, two children (K. Y. and I. K.) showed positive balance, but the other three children (I. Y., T. D. and S. T.) did not. One of these (S. T.) had a cold during this period.

Finally, for the two children who reached positive balance, the amount of tryptophan only was decreased by half (0.25 gm), and for the three children who had not reached positive balance, the essential amino acid mixture was increased by three times the minimal requirements defined by Rose et al. ('55) keeping the total nitrogen amount at a 12-gm level.

It was found that tripling the amount of essential amino acids was adequate to maintain the positive balance (except for S. T.), and that 0.25 gm of tryptophan was also adequate for children, as long as other essential amino acids were kept at twice the amount of the minimal requirement of adults. Of course, if tryptophan was totally withdrawn from the amino acid mixture, a negative balance would result as seen in the first experiment. In all children

the excretion of creatine and creatinine remained almost at a constant level.

CONCLUSIONS

1. On the basis of nitrogen balance, it has been estimated that the school children require two or three times as much essential amino acid mixture as adults, if the pattern of Rose et al. ('55) for essential mixture is not unbalanced for children.

2. The total nitrogen is not sufficient at a level below 10 gm for children 12 to 14 years old.

3. The requirement for tryptophan is almost the same as that found by Rose et al. ('54).

4. When the pattern set by Rose et al. ('55) is modified for children, the total amount of essential amino acids required will differ considerably from the amount mentioned above.

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The Biological Activity of 6-Ethyl-9-(1′-D-Ribityl)-Isoalloxazine[1]

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Several homologs of riboflavin have been synthesized and tested for biological activity. Of these a few have been found to be active as vitamin-like substances for certain species and a few others as inhibitor-like substances for certain species. Some have been found to stimulate growth in the riboflavin-deficient rat, for example, 6-methyl- (Karrer and Quibell, '36; Kuhn et al., '37), 7-methyl- (Karrer et al., '35), 6-ethyl-7-methyl- (Karrer and Quibell, '36; Lambooy, '58a), and 6-methyl-7-ethyl-9 - (1′-D - ribityl) - isoalloxazine (Lambooy, '58a). All of these plus 6,7-diethyl - 9 - (1′-D - ribityl) - isoalloxazine (Lambooy, '51) have been found to support growth and acid production in *Lactobacillus casei* (Snell et al., '39).[2]

In 1936, Karrer and Quibell ('36) synthesized 7 - ethyl - 9-(1′-D-ribityl) - isoalloxazine and reported it to have no growth promoting activity for the rat when used as the sole flavin, but to stimulate growth of the riboflavin-deficient rat when the animal received inadequate quantities of riboflavin (Karrer, '36).

Since the 6-methyl-flavin and the 7-methyl-flavin described above have less than one-half the activity of the 6,7-dimethyl-flavin (riboflavin) and since 6,7-diethyl-flavin is an inhibitor of riboflavin in the rat (Lambooy and Aposhian, '52), it would seem consistent that the activity of 7-ethyl-flavin might be expected to lie somewhere between that of the active 7-methyl-flavin and the inhibitor 6,7-diethyl-flavin. Such a position might be interpreted as "inactivity." It would be of interest to study the biological properties of the isomeric 6-ethyl-9-(1′-D - ribityl)-isoalloxazine to see if it also is inactive or whether it possesses a low level of vitamin-like or inhibitor activity. We have prepared this new flavin by two independent routes (Aposhian and Lambooy, '54 and Lambooy, '58b) and have studied its properties in *L. casei* and in the riboflavin-deficient rat.

The purity of the material has been established to the satisfaction of the authors by elemental analysis, spectrophotometric analysis and exhaustive chromatographic analysis.

METHODS

Acid production by L. casei. The quantity of lactic acid produced in the routine method for the microbiological assay of riboflavin (Assoc. of Vitamin Chemists, '51) was measured, using in one case graded increments of United States Pharmacopeia Riboflavin Reference Standard and in the other, equal amounts of analytically-pure 6-ethyl-flavin. This procedure revealed that the activity of the homolog was of the order of 5% of that of riboflavin. The levels of the 6-ethyl-flavin were repeatedly increased until they were sufficient to produce a "standard" curve which was similar to the one simultaneously obtained for riboflavin. *Lactobacillus casei* 7469[3] was used.

Rat growth. The first stage of this study made use of weanling male Wistar

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[1] This investigation was supported in part by a research grant no. CY 2940 from the National Cancer Institute of the Public Health Service.
[2] Lambooy, J. P. 1957 Synthesis and activity for *Lactobacillus casei of* 6-ethyl-(methyl)-7-methyl- (ethyl) -9-(1′-D-ribityl) - isoalloxazines. Federation Proc., *16:* 208 (abstract).
[3] American Type Culture Collection, Washington, D.C.

rats which were free of vermin and kept in individual cages in a temperature-controlled room (24°C). All animals were placed on a riboflavin-deficient diet similar to that used before (Lambooy and Aposhian, '52) except that thiamin chloride hydrochloride and pyridoxine hydrochloride levels were raised to 20 mg, calcium pantothenate to 60 mg, biotin to 1.8 mg, cyanocobalamine to 40 μg, and choline chloride to 1.5 gm per kg of diet. The animals were fed this diet ad libitum until not only had they ceased to gain weight but were losing weight. When they were deficient, groups of three or 4 animals were given 10, 20, 40, 80 and 500 μg of the 6-ethyl-flavin in 0.5 ml of water by stomach tube each day for 28 days immediately before being given food. A control group of 9 animals was given only the 0.5 ml of water whereas other groups of three or 4 animals received graded levels of riboflavin; only the 2-μg and 4-μg per day groups are used for comparison.

Riboflavin inhibition. To obtain experimental animals of low and relatively uniform riboflavin stores, we fed 52 weanling female Wistar rats the above diet except that riboflavin was added at the level of 5 μg per gm. When these females reached 180 gm, which they did more slowly than others fed the same diet except that 20 μg of riboflavin was added per gm, they were bred. Of the 52 females started, two died, one failed to reach 180 gm, two failed to have young, and only 16 of the remaining 47 which had litters were able to raise some of their young to the weanling stage. The pups of the remaining 3 females all died during the lactation period as did some of the pups of some of the above 16 females. When available, weanling males of these litters were paired as littermates into two groups. They were fed the riboflavin-deficient diet for three weeks at which time they had all become deficient although a few were not actually losing weight. As each pair reached the deficient state, one, as a member of the experimental group, received a suspension of 2 mg of 6-ethyl-flavin in 0.5 ml of 6% of gum acacia solution by stomach tube immediately before being fed, and the other, as a member of the control group, received 0.5 ml of acacia solution by the same means at the same time each day for 28 days. Ten animals constituted each group.

When the results of the administration of 2 mg of 6-ethyl-flavin or none became available, it was apparent that a second pair of supplements must be studied. Again, as littermate pairs (10 total) became available and then deficient, one of each pair was given 2 mg of 6-ethyl-flavin *plus* 1 μg of riboflavin and the control was given only the 1 μg of riboflavin, by the procedures outlined earlier. Again, the results indicated that further paired supplements must be studied, and this time one member of each pair (11 total) was given 2 mg of 6-ethyl-flavin *plus* 5 μg of riboflavin while the control received only the 5 μg of riboflavin as described above.

Some unpaired males and several females were sacrificed at the time their littermates were placed on the experiment. These animals (38 animals) were analyzed for total flavin content by the routine microbiological procedure.

Preparation of animals for analysis. The littermates which were assayed for riboflavin were processed as follows. The animal was weighed, killed by an overdose of ether, and the stomach and cecum cleaned of contents. The entire animal was then passed through a fine meat grinder and the whole of the product quantitatively transferred to a flask by the aid of water, immediately frozen and eventually lyophylized. The tissue was finally dried to constant weight (8 mg or less change between two successive weighings) in a vacuum desiccator. Each animal was then hydrolyzed in approximately 50 ml of 0.1 N HCl per gm of dry tissue for 30 minutes at 15 p.s.i., the solution cooled, adjusted to pH 4.5, diluted to one l and filtered. These filtrates were assayed for riboflavin by the usual microbiological procedure and the results expressed as micrograms of riboflavin per gram of fresh and of dry tissue with the standard errors.

RESULTS

Acid production by L. casei. When the quantities of 6-ethyl-flavin and riboflavin

used for the preparation of their respective standard curves were the same, all titration values for the acid produced in the tubes containing the graded levels of 6-ethyl-flavin (0.05 to 0.30 μg) fell between the blank and the value for the lowest level (0.05 μg) of riboflavin. This low level of activity for 6-ethyl-flavin (estimated at about 5%) necessitated repeated increases in the level of the homolog until at concentrations of from two to 10 μg per tube the acid production was essentially the same as that found for riboflavin throughout the concentration range of 0.05 to 0.25 μg per tube (fig. 1). The homolog's activity is, therefore, only 3% of that found for riboflavin. No inhibitory properties were found when *L. casei* was grown in a medium containing 0.05 μg of riboflavin plus quantities as high as 450 μg (1:9000) of the homolog per 10 ml of solution. The acid production in even this extreme case exceeded that found in the tubes containing the corresponding quantity of riboflavin alone.

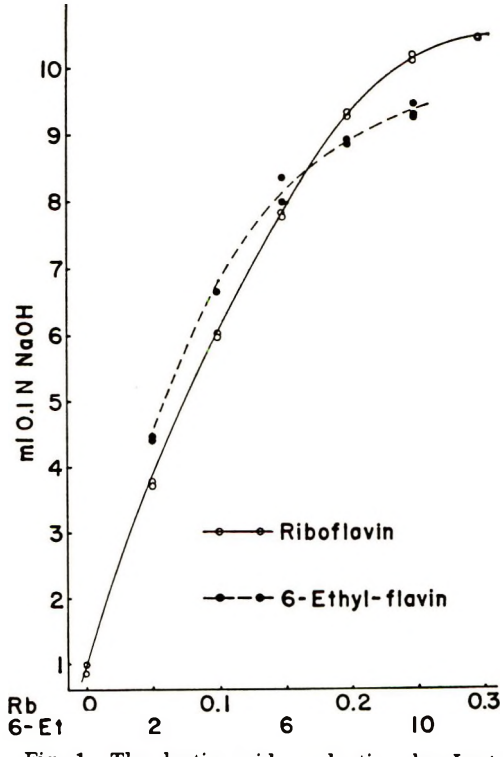

Fig. 1 The lactic acid production by *Lactobacillus casei* 7469 in the presence of riboflavin (0 to 0.3 μg per tube) ———, and 6-ethyl-flavin (2.0 to 10.0 μg per tube) — — —.

Rat growth. The administration of the 6-ethyl-flavin at levels of 10 to 80 μg per day showed that the homolog had no growth promoting activity in the riboflavin-deficient rat. The growth curves for these 4 groups clustered about that for the deficient controls and are, therefore, considered as a single group ($P = 0.324$). One third (3/9) of the deficient control group failed to survive the 28-day test period; the average weight gain was one gm and the range was − 7 to + 16 gm. Of the combined 10 to 80 μg per day groups, one third (4/12) of the animals failed to survive the 28-day test period; the average weight gain was 6 gm and the range was − 9 to + 17 gm.

The administration of the 6-ethyl-flavin at the level of 500 μg per day resulted in failure of three of the animals to survive the test period; the survivor showed a net weight loss of 2 gm. Groups of animals were also maintained on graded levels of riboflavin but the only two groups of interest for comparative purposes were: the group receiving 2 μg of riboflavin which had an average gain in weight of 14 gm (vs deficient controls $P = 0.051$), the range was 10 to 18 gm; the group receiving 4 μg of riboflavin which had an average gain of 34 gm (vs 2 μg riboflavin $P = 0.014$), the range was 28 to 42 gm when the material was administered under the same regimen as that described for the 6-ethyl-flavin.

Riboflavin inhibition. The suggestion that 500 μg per day of the 6-ethyl-flavin might be toxic for the riboflavin-deficient rat, possibly as an antagonist of riboflavin, prompted us to produce test animals of relatively low and uniform tissue stores of riboflavin. The littermates of the experimental animals which were assayed for total riboflavin, and incidentally for water content, yielded the following information. Lyophylization of the ground animals gave an excellent product for handling and assay. The percentage of dry substance was 32.1 ± 0.3.[4] The riboflavin content was 3.92 ± 0.07 μg per gm of wet

[4] The ± values given are standard error.

weight and 12.1 ± 0.3 μg per gm of dry tissue. It is to be emphasized that these values pertain to the weanling young of females who themselves had been raised from weaning on a diet which contained only 5 μg of riboflavin per gm of diet and presumably also represent the levels of riboflavin in the animals subjected to the experimental procedure.

After the paired littermates were fed the riboflavin-deficient diet for three weeks their weights had levelled off and in a majority some weight loss had occurred. The first study (pair 1, fig. 2) compared 2 mg of 6-ethyl-flavin with no flavin. Neither of the two groups showed any gain in weight. None of the deficient control group died whereas 80% (8/10) of those receiving the 6-ethyl-flavin failed to survive, deaths occurred between days 9 and 25. At the end of the 28-day test period all surviving animals (10 from deficient control groups and two from 6-ethyl-flavin group) were given a suspension of 2 mg of riboflavin in 0.5 ml of 6% gum acacia solution each day for 10 days. The average weight gained by the deficient control group was 72 gm; that for the two 6-ethyl-flavin animals was 64 gm (P = 0.22).

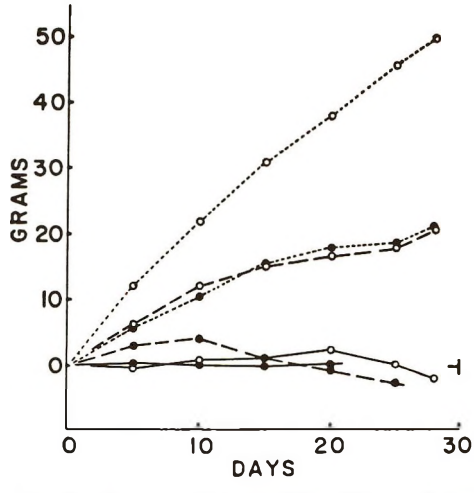

Fig. 2 Growth of rats fed diets containing various combinations of flavin supplements. Pair 1, ○—○ flavin deficient control, ●—● 2 mg 6-ethyl-flavin; pair 2, ○ - - - ○ 1 μg riboflavin control, ●- - -● 2 mg 6-ethyl-flavin *plus* 1 μg riboflavin; pair 3 ○ . . . ○ 5 μg riboflavin control, ●- · -● 2 mg 6-ethyl-flavin *plus* 5 μg riboflavin.

The second study (pair 2, fig. 2) compared 2 mg of 6-ethyl-flavin *plus* 1 μg of riboflavin per day with a control group receiving 1 μg of riboflavin per day. None of the control group died and the average weight gain of this group was 21 ± 1 gm. Forty per cent (4/10) of the animals receiving the 2 mg of 6-ethyl-flavin *plus* 1 μg of riboflavin died between days 16 and 20. This latter group showed no weight gain (3 ± 6 gm), was different from its control group (P = 0.009) but not from the negative control group of pair 1.

The third study (pair 3, fig. 2) compared 2 mg of 6-ethyl-flavin *plus* 5 μg of riboflavin with a control group receiving 5 μg of riboflavin per day. All 11 animals of each group survived. The first group showed an average weight gain of 21 ± 3 gm; the growth curve being essentially superimposable (P = 1.00) upon the growth curve of the control group of pair 2. The animals receiving 5 μg of riboflavin per day without the isomer showed an average weight gain of 51 ± 3 gm and differed from the group receiving the isomer (P = better than 0.00001, t = 6.45) and from the group receiving 1 μg of riboflavin in pair 2 (P = better than 0.00001, t = 6.76).

All animals which received 2 mg of 6-ethyl-flavin or 2 mg of 6-ethyl-flavin *plus* 1 μg of riboflavin and survived but failed to gain weight (8 animals) showed an average loss of 3 gm. The weight gain shown by the animals receiving 2 mg of 6-ethyl-flavin *plus* 5 μg of riboflavin was 21 gm or a difference of 24 gm. The difference between the latter group and the growth shown (51 gm) by the controls on 5 μg of riboflavin was 30 gm. The 5 μg of riboflavin almost halved the inhibition due to 2 mg of 6-ethyl-flavin, or 2 mg of 6-ethyl-flavin almost halved the growth promoting properties of 5 μg of riboflavin. This relationship indicates that 6-ethyl-9-(1′-D-ribityl)-isoalloxazine is a weak antagonist of riboflavin with an inhibition index of approximately 400.

DISCUSSION

Little need be said concerning the activity of 6-ethyl-flavin for *L. casei*. Three

homologs of riboflavin are nearly as potent as the vitamin for this organism throughout limiting concentration ranges; they are 6-ethyl-7-methyl- (Snell and Strong, '39)[5] 6-methyl-7-ethyl-,[6] and 6,7-diethyl-9-(1′-D-ribityl)-isoalloxazine (Lambooy, '51). Two other homologs have about 50% of the potency of riboflavin under the same conditions; they are 6-methyl- and 7-methyl-9-(1′-D-ribityl)-isoalloxazine (Snell and Strong, '39). In 6-ethyl-9-(1′-D-ribityl)- isoalloxazine we find an isomer of extremely low activity. Rarely does one find such a wide range of activity among the homologs of a biologically active compound. If the structure of 6,7-dimethyl-flavin (riboflavin) is altered by the displacement of one or both of its methyl groups by ethyl groups, it retains approximately 90% of its activity for *L. casei*. If the vitamin loses one of its methyl groups, it loses about 50% of its activity, and if it loses both, it loses all of its activity. The activity found for the 6-ethyl-flavin is the first instance of activity being associated with a mono-ethyl isomer of riboflavin.

When it appeared that the 6-ethyl-flavin was a weak antagonist of riboflavin for the rat, it became important to find a means of obtaining experimental animals of relatively low and uniform stores of riboflavin. Another study in which we are engaged suggested that the young born of females which had been raised from weaning on a diet containing 5 μg per gm of riboflavin would serve our purpose. At this level of riboflavin, as indicated above, the growth rate of these females is depressed and reproduction capacity is sharply diminished.

There are many points of common interest between this study and the excellent one reported recently by Bessey and coworkers ('57). These investigators found that the average carcass (devoid of liver, blood and hair) content of riboflavin of a well-nourished 55-gm rat was about 3.8 μg per gm of fresh tissue. The average content which we found to be characteristic of the *whole* animal of an average weight of 46 ± 1 gm was 3.9 μg per gm of fresh tissue. While it is not possible to reconstruct a whole animal from the data presented by Bessey et al., we find that Donaldson ('24) has shown that a 55-gm rat will have a liver weight and a blood volume of about 3.8 gm and 3.6 gm respectively. Bessey and co-workers found 16.2% of protein in the liver of their weanling rats. From these data we can sum up a 48-gm carcass plus 3.8 gm of liver plus 3.6 gm of blood, containing 181 μg (carcass) plus 97 μg (liver) of riboflavin or 278 μg per 55 gm which is equivalent to 5.1 μg per gm of fresh tissue. This value agrees well with other data reported by Bessey et al. (their table 1, series 1) where 8 animals of 40-gm average weight had an average riboflavin content of 5.8 μg per gm and later (their footnote 4) where two similar average tissue levels were reported to be 5.1 and 5.2 μg per gm of fresh tissue.

Our animals with an average tissue content of 3.9 μg per gm must have had diminished stores of riboflavin, and again we find the Bessey et al. report (their table 1, series 2) showing the average value for 5 animals of an average weight of 71 gm to be, *following two weeks on a riboflavin-deficient diet*, 3.36 μg per gm of fresh tissue. Furthermore, these investigators report that when both sexes were fed suboptimal quantities of riboflavin, the female tissues contained higher levels of the vitamin than those of the male. Our value of 3.9 μg was obtained from a group made up of 92% females; since all of our experimental animals were males, it seems reasonable to assume that their average tissue level was somewhat lower.[7]

We are at a loss to reconcile the finding by Bessey et al. that "maximum riboflavin concentrations are obtained with intakes of about 40 μg per day" with our observations that 5 μg of riboflavin per gm of food is inadequate for a normal growth rate for all and for reproduction for a majority (69%) of our breeder females for the inhibition studies. To obtain 40 μg per day these animals would be required to eat only 8 gm of food a day.

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[5] See footnote 2.

[6] See footnote 2.

[7] The three males which made up the 8% of the above group had, however, an average value of 3.92 μg/gm.

Our values for the water content of weanling rats appears to be lower than most of the values reported in the literature. This may be due to one or two factors, the first of which is technique. Formerly animals were dried to constant weight in an oven heated to 100°C or above. Our procedure of non-destructive lyophylization does not measure as "water" loss volatile fatty acids and other volatile products resulting from heat destruction of the tissues. The animals used for the assay of total flavin were somewhat malnourished relative to the customary "well nourished" animal and this may have some influence on the water content.

It is interesting that the 6-ethyl-9-(1′-D-ribityl)-isoalloxazine is similar to its isomer 7-ethyl-9-(1′-D-ribityl)-isoalloxazine in that neither has growth promoting activity for the riboflavin-deficient rat. It has been claimed that the 7-ethyl isomer stimulates growth when it is administered to rats receiving inadequate levels of riboflavin (Karrer, '36). The 6-ethyl isomer is a mild inhibitor under these circumstances.

SUMMARY

It has been found that 6-ethyl-9-(1′-D-ribityl)-isoalloxazine has approximately 3% of the activity of riboflavin when used as the sole flavin in the nutrition of *Lactobacillus casei*.

This isomer of riboflavin has been found to be devoid of growth promoting activity in the riboflavin-deficient rat. It is a low-potency antagonist of riboflavin with an inhibition index of approximately 400.

ACKNOWLEDGMENT

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The Control of Filicidal Cannibalism by Vitamin B_{12}

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It has been shown that filicidal cannibalism among female albino rats fed a modified Bills diet ('31) can be greatly reduced by the addition of 3% of whole liver powder to the diet (Hankin, '58). A one-per cent level of liver was ineffective. Also, the inclusion of this supplement in the diet of the mothers resulted in increased weaning weights of the young.

Although liver powder was effective in diminishing filicidal cannibalism, the nature of the active component in this nutrient was not ascertained. This investigation was made in order to determine whether vitamin B_{12} is involved.

EXPERIMENTAL

Weanling female albino rats of the Osborne-Mendel strain were fed either the modified Bills diet (Hankin, '58) or one of two test diets. These contained either 15 or 30 μg of added crystalline vitamin B_{12} per kg.[1] The lower level gave a calculated total concentration of vitamin B_{12} equivalent to that contained in 3% of dried liver (Elvehjem, '50). Since the control diet contained 7.5 μg of B_{12} per kg,[2] the final concentration in the two test diets was, respectively, 22.5 and 37.5 μg per kg.

The experimental procedures have been described previously in detail (Hankin, '58).

RESULTS

Filicidal cannibalism by female albino rats fed a modified Bills diet can be controlled by the addition of either 15 or 30 μg of vitamin B_{12} per kg of basal diet. No difference was noted between the two test levels of the vitamin, but there was a marked difference between the test levels and the control. Table 1 shows the values obtained.

As the vitamin B_{12} supplement was effective in the control of filicidal cannibalism, a statistical evaluation of its contribution was made by comparing these data with the results previously obtained by feeding a supplement of whole liver powder (Hankin, '58). Since a one per cent level of liver was ineffective in the control of this behavior, the data obtained at this level were combined for the statistical analysis with the values obtained when the control diet alone was fed. Also, as both test levels of vitamin B_{12} were equally effective, these data were also combined. The differences between the observed and expected frequencies in the two four-fold tables were totaled and their sum squared and divided by the sum of their variances.[3] This analysis is summarized in table 2.

The discrepancy between the two tables of χ^2, namely 0.135, is so low, it may be concluded that there is no difference between the effectiveness, in the control of filicidal cannibalism, of 3% of whole liver powder or of either 15 or 30 μg of added vitamin B_{12} per kg of diet. Furthermore, the results show that vitamin B_{12} is the active principle in the dried liver which has been found to be effective (Hankin, '58).

The addition of vitamin B_{12} to the diet of the mother rats has resulted in an increase in the weaning weights of the young from an average of 36.3 gm for the controls to an average of 43.0 gm. A statistical evaluation of these data has shown a positive correlation between birth weight and weaning weight of progeny when the mothers received the supplement. How-

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[1] Generously supplied by Dr. C. R. Szalkowski, Merck Sharp and Dohme, Rahway, New Jersey.

[2] Estimated value.

[3] Suggested by Dr. A. T. James, Associate Professor of Mathematics, Yale University, New Haven, Conn. in a personal communication to Dr. C. I. Bliss, Biometrician, The Connecticut Agricultural Experiment Station, New Haven, Conn.

TABLE 1

Control of filicidal cannibalism by addition of vitamin B_{12} to the Bills diet

Diet	No. of litters	No. of litters eaten	% Eaten
Control	58	23	39.7
15 μg Vitamin B_{12} supplement per kg	43	10	23.3
30 μg Vitamin B_{12} supplement per kg	44	9	20.5

TABLE 2

Statistical comparison of data obtained using whole liver powder[1] and vitamin B_{12} in the control of filicidal cannibalism

	Liver No. of litters			Vitamin B_{12} No. of litters		
	Remaining	Eaten	Total	Remaining	Eaten	Total
Control	78	41	119	35	23	58
Effective dose	35	5	40	68	19	87
Total	113	46	159	103	42	145

Analysis

	D.F.	χ^2
Combined effect liver + vitamin B_{12}	1	12.251
Discrepancy	1	0.135
Total of separate effects	2	12.386
Combined χ^2 corrected for continuity	1	11.311

[1] Hankin ('58).

ever, the presentation here of the detail of this analysis is not warranted.

As had previously been noted when a liver supplement was employed (Hankin, '58), the usefulness of progeny from mothers fed the vitamin B_{12} supplement was not in any way impaired for the bioassay of vitamin D.

DISCUSSION

An interesting aspect of these observations is that the control diet appears to contain an amount of vitamin B_{12} sufficient for daily maintenance, as the daily requirement has been shown to be about 0.8 μg per kg of body weight (Emerson, '49). Although the Bills diet has been shown to be slightly inferior to other diets for reproductive efficiency, it is adequate for growth (Hubbell, '54). In any event, the control diet supplemented with one per cent of dried liver contains more than a sufficient supply of vitamin B_{12}, but this too was ineffective in controlling canibalism. Clearly the requirement for vitamin B_{12} by the rat for daily maintenance differs from the requirement during pregnancy and lactation (Jaffe, '56).

If cannibalism was entirely a matter of the vitamin B_{12} requirement, then second and third litters would be eaten more frequently since the vitamin B_{12} in the body would presumably be more nearly depleted. However, consistent results were not obtained since there was no definite pattern as to the litters in which cannibalism occurred. A more likely explanation is that there is an interrelationship between vitamin B_{12} and some other nutrient in the diet which mediates the requirement for this vitamin. Rice et al. ('58) have noted that sorbitol enhances absorption of vitamin B_{12} in pregnant rats. It has also been reported that a high-fat diet leads to an increase in the requirement for B_{12} by the chick (Fox et al., '56). Diets high in fat or high in both fat and protein have also been shown to increase the requirement for vitamin B_{12} in the rat (Peterson and Register, '58). This does not seem to apply to the Bills diet which is not notably high in either protein (18%)

or fat (8%). It appears, therefore, that fat per se is not the component in the Bills diet which brings about the increased requirement for vitamin B_{12}. A possibility does exist that there is a relationship between the requirement for vitamin B_{12} and the type of fat in the diet. Most diets for rats contain either corn or soybean oil. In the Bills diet, 67% of the fat is butterfat. This aspect of the problem of filicidal cannibalism suggests further study.

Although vitamin B_{12}, when fed to mother rats, led to an increase in the weaning weight of the young, it was not as effective as the desiccated liver supplement since liver clearly contains many additional nutrients beneficial for growth.

SUMMARY

The effect of adding crystalline vitamin B_{12} to the Bills breeding diet, in order to control filicidal cannibalism, has been investigated. A level of vitamin B_{12} comparable to that found in a diet supplemented with 3% of desiccated liver has been shown to be effective. Statistical analysis of the data has shown that it is the vitamin B_{12} component of the liver which provides the necessary factor in the control of filicidal cannibalism.

ACKNOWLEDGMENTS

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Blood Volume and Serum Protein in the Deoxypyridoxine-Fed Rat During Pregnancy[1,2]

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Recent work in this laboratory has centered on changes in the vitamin B_6-deficient rat during pregnancy, particularly those changes which might be related to toxemia. In a previous report (Brown and Pike, '60) it was shown that while blood pressure remained normal in the pregnant and non-pregnant deficient animal, there was a tendency toward water retention in the deficient state.

The present report is a continuation of the previous work and deals with changes in blood volume, hematocrit, hemoglobin, total serum protein and serum protein fractions in deoxypyridoxine-fed and control pregnant animals. In order to separate the normal changes during pregnancy from those produced by the antagonist, determinations were made also on non-pregnant animals.

EXPERIMENTAL PROCEDURE

Female rats of the Sprague-Dawley strain which had been reared on a laboratory stock diet were placed on the experimental diets when they had reached a weight of 200 gm. Details of the dietary treatment and experimental conditions have been described elsewhere (Brown and Pike, '60).[5] Pregnant and non-pregnant animals were divided into three diet groups: (1) non-depleted controls, fed the basal ration containing 0.8 mg % of pyridoxine; (2) depleted controls, fed the basal ration containing 2.0 mg % of deoxypyridoxine for a minimum of 6 days prior to mating and the pyridoxine-containing diet for the three week gestational period; and (3) deficient groups, fed the deoxypyridoxine-containing diet both prior to and during the gestational period. Vaginal smears were examined daily and animals were mated at the appropriate time in the estrous cycle.

Blood analyses were made on an ultramicro scale in order to avoid excessive bleeding of the animals. Blood was collected from the tip of the tail into capillary tubes. No more than 0.3 ml was taken at a time.

Hematocrit determinations were made by the micro hematocrit technique described by Strumia et al. ('54). Plasma volume was determined by an ultramicro adaptation of the Evans blue dye dilution method described by Loring ('54). An injection of 0.1 ml of 0.5 % Evans blue dye was made into the caudal vein and blood was collected from the tip of the tail two minutes following injection for the determination of plasma volume. Preliminary work indicated that a two-minute interval permitted complete mixing of the dye and the blood. Blood volume was calculated from hematocrit and plasma volume. Hemoglobin was estimated from the specific gravity of whole blood and serum using the method of Van Slyke et al. ('50).

Total serum protein was determined from specific gravity of serum using a gradient tube as described by Lowry and Hunter ('45). Blood protein fractions were determined by paper electrophoresis using a horizontal strip apparatus as described

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by Grassman and Hannig ('52). Veronal buffer pH 8.6 with an ionic strength of 0.03 was used. A serum sample of 4.3 µl was applied and separations were made at 250 volts for 5 hours. Strips were dyed with amido black. Tracings of the strips were made using a densitometer. The tracings indicated that complete separation of albumin and alpha$_1$ globulin was not effected by this procedure. It was necessary, therefore, to complete the curves and drop a line at their intersection for estimation of the two components individually. Fractions were determined as percentage of the total area and the concentrations calculated from the concentration of total serum protein.

Plasma volume and hematocrit determinations were made on the first, 8th, 15th and 21st days of experiment. Total protein of serum, serum protein fractions and hemoglobin determinations were made only on the first and 21st days of experiment.

The data were subjected to an analysis of variance with appropriate corrections for disproportionality among the groups.

RESULTS AND DISCUSSION

Blood volume increased significantly by the end of the gestation period in pregnant animals fed pyridoxine (table 1). This was expected since blood volume is known to increase during pregnancy in most species. Little change in total blood volume was observed in any of the non-pregnant groups and there was a gradual decrease in blood volume in the pregnant group fed deoxypyridoxine. Only half of these animals were able to carry young to term, however. When those carrying young are separated from those unable to maintain pregnancy due to the deleterious effect of the antimetabolite, it is evident that the reduction in total blood volume was greater for the animals failing to carry young successfully and these values weighted the average for the entire group (table 2).

The increase in total blood volume observed in the pregnant animals in this study does not appear to be related directly to gain in body weight. If it were, blood volume as percentage of body weight should remain approximately the same in all animals as has been reported by Bond ('48). Per cent blood volume decreased somewhat in all animals but the greatest decrease was observed in the pregnant control groups in which the gain in both total blood volume and body weight were greatest.

That the increased total blood volume in the pregnant animals fed pyridoxine is due primarily to dilution is indicated by a comparison of plasma volume and hematocrit levels (fig. 1). In control pregnant animals, total plasma volume increased in contrast with that of the non-pregnant controls. It is interesting that even in the deoxypridoxine-fed animals total plasma volume increased slightly in animals carrying young, whereas plasma volume for the animals unable to complete their pregnancies follows the trend observed in the deoxypyridoxine-fed non-pregnant animals. Hematocrit levels decreased in all pregnant animals but were lowest in the deoxypyridoxine-fed group. The failure of the deoxypyridoxine group to show substantial increases in plasma volume in the face of a decreasing hematocrit indicates the diminishing hematocrit is not the result of hemodilution only, as in the other pregnant animals, but rather is evidence of an actual decrease in red cells.

Hematocrit levels decreased also in the deoxypyridoxine-fed non-pregnant animals but total plasma volume increased only slightly above the initial level. Total blood volume remained approximately the same throughout the experiment. Therefore, the reduction in hematocrit, as in the pregnant deoxypyridoxine-fed group, represents an actual reduction in red cells. The data for hemoglobin substantiates this conclusion.

Hemoglobin levels followed the same pattern observed in hematocrit levels (table 3). It would seem that the decrease in hemoglobin observed in pregnant deoxypyridoxine-fed animals was associated chiefly with the deleterious effect of the antagonist. The small increase in total plasma volume could not produce a state of hemodilution great enough to account for the reduction in hemoglobin observed in these animals. Hemoglobin levels were reduced significantly ($P = 0.001$) in non-pregnant deoxypyridoxine-fed animals also.

TABLE 1

Blood volume of pregnant and non-pregnant control and deoxypyridoxine-fed rats

Dietary treatment	Non-pregnant				Pregnant			
	Day				Day			
	1	8	15	21	1	8	15	21
	Total blood volume (ml)							
Non-depleted, 0.8 mg% pyridoxine	10.99 ± 0.76[2]	11.00 ± 1.28	11.88 ± 1.56	11.50(7)[1] ± 1.08	11.45 ± 1.52	11.71 ± 1.45	11.32 ± 1.12	12.96(7) ± 1.37
Depleted, 0.8 mg% pyridoxine	11.20 ± 1.65	12.02 ± 0.72	11.89 ± 1.52	11.20(11) ± 1.27	10.36 ± 1.37	10.82 ± 1.72	11.91 ± 1.79	13.61(8) ± 1.62
Depleted, 2.0 mg% deoxypyridoxine	10.02 ± 0.77	10.80 ± 1.18	9.70 ± 1.04	9.96(9) ± 1.23	10.93 ± 0.96	10.85 ± 1.23	10.53 ± 0.84	10.17(8) ± 1.42
	Ml per 100 gm body weight							
Non-depleted, 0.8 mg% pyridoxine	5.38 ± 0.37	5.02 ± 0.66	5.10 ± 0.64	4.99 ± 0.45	5.70 ± 0.83	5.16 ± 0.65	4.50 ± 0.34	4.20 ± 0.22
Depleted, 0.8 mg% pyridoxine	5.63 ± 0.88	5.04 ± 0.40	4.91 ± 0.73	4.69 ± 0.43	5.07 ± 0.63	4.74 ± 0.70	4.75 ±0.84	4.59 ± 0.41
Depleted, 2.0 mg% deoxypyridoxine	5.26 ± 0.79	5.67 ± 0.32	5.36 ± 0.69	5.53 ± 0.82	5.64 ± 0.62	5.53 ± 0.73	5.46 ± 0.41	5.17 ± 0.60

[1] Number of animals in group.

[2] Standard error of the mean.

TABLE 2

Blood volume of pregnant group fed deoxypyridoxine

	No. animals	Day 1	Day 8	Day 15	Day 21
		Total blood volume (ml)			
With litters	4	11.09 ± 1.15[1]	10.44 ± 0.91	10.85 ± 0.93	10.76 ± 1.61
Without litters	4	10.77 ± 0.69	11.26 ± 1.37	10.29 ± 0.69	9.58 ± 0.89
		Ml per 100 gm body weight			
With litters	4	5.62 ± 0.72	5.20 ± 0.60	5.33 ± 0.17	5.00 ± 0.75
Without litters	4	5.67 ± 0.50	5.87 ± 0.70	5.56 ± 0.50	5.34 ± 0.31

[1] Standard error of the mean.

The simultaneous determination of blood volume allowed an estimation of the total circulating hemoglobin (table 3). It is clear that, although hemoglobin concentration at term is relatively low in pregnant animals fed pyridoxine, the absolute quantity is not diminished. This was not true for either the pregnant or non-pregnant animals fed deoxypyridoxine as the data for hematocrit and plasma volume appeared to indicate. In the deficient animals both the relative and absolute amounts of hemoglobin are reduced. These observations are in agreement with earlier work from this laboratory (Pike and Brown, '59) and suggest that hemoglobin and red cell synthesis are decreased in vitamin B_6 deficiency.

It has been suggested that hemoconcentration may be a consequence of vitamin

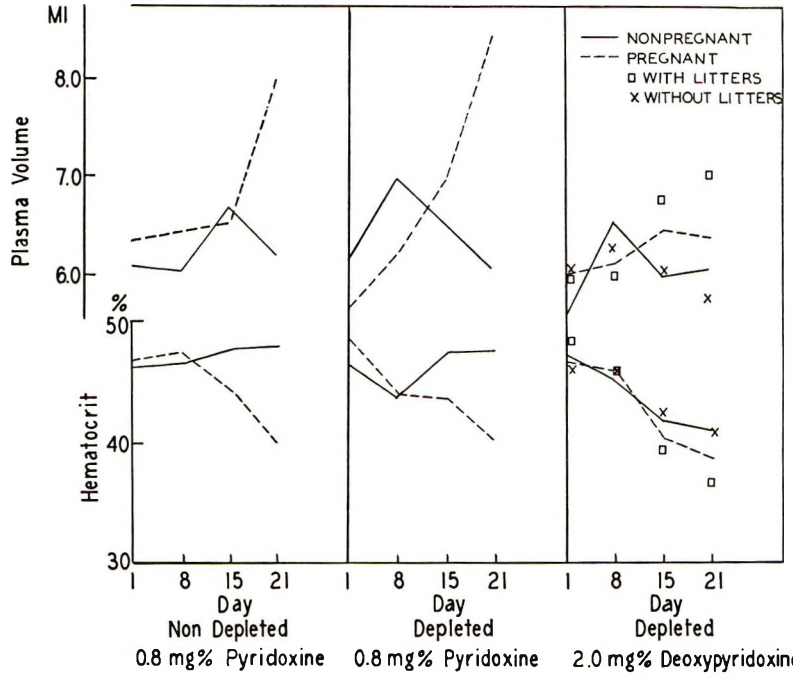

Fig. 1 Plasma volume and hematocrit of pregnant and non-pregnant control and deoxypyridoxine-fed rats.

TABLE 3

Hemoglobin of pregnant and non-pregnant control and deoxypyridoxine-fed rats

Dietary treatment	Non-pregnant				Pregnant			
	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21
	gm%	*gm%*	*gm*	*gm*	*gm%*	*gm%*	*gm*	*gm*
Non-depleted, 0.8 mg% pyridoxine	16.6 ± 0.7[2]	17.0 ± 1.8	1.82 ± 0.06	1.95(7)[1] ± 0.11	16.5 ± 1.0	13.7 ± 0.7	1.88 ± 0.12	1.77(7) ± 0.09
Depleted, 0.8 mg% pyridoxine	17.2 ± 0.7	16.2 ± 0.8	1.94 ± 0.12	1.82(11) ± 0.08	17.2 ± 0.7	13.8 ± 0.8	1.79 ± 0.11	1.88(8) ± 0.09
Depleted, 2.0 mg% deoxypyridoxine	17.3 ± 0.8	14.3 ± 2.1	1.74 ± 0.14	1.43(9) ± 0.11	17.3 ± 0.8	13.8 ± 1.6	1.89 ± 0.06	1.40(8) ± 0.09

[1] Number of animals in group.
[2] Standard error of the mean.

B_6 deficiency in the rat (Pike and Brown, '59). In the present study, both hemoglobin and hematocrit levels were significantly higher ($P = 0.01$) on the first day of experiment in depleted animals than in non-depleted animals. Conversely, total plasma volume tended to be smaller in depleted animals although the difference was not statistically significant. After the first week, neither hematocrit, hemoglobin nor plasma volume appeared to have any significant relationship to prior depletion. This effect of hemoconcentration in the deoxypyridoxine-fed animal occurs rather early before both hemoglobin and hematocrit levels begin to fall.

The significance of the apparent tendency toward hemoconcentration may be related to the serum electrolyte imbalance reported by Hsu et al. ('58). Some aspects of this problem are currently being investigated in this laboratory.

In toxemia of pregnancy both blood and plasma volumes may decrease (Berlin et al., '52). The downward shift in total blood volume in deoxypyridoxine-fed animals in this study is suggestive of the changes occurring in complications of pregnancy. However, since diminishing plasma volumes occurred primarily in the animals unable to maintain pregnancy, no definite analogy between the reduction in total plasma volume in this study and that occurring in human toxemia of pregnancy can be made.

The decrease in the concentration of total serum protein observed in the pregnant animals in this study has been found repeatedly in pregnant animals and is attributed to the increase in total plasma volume which normally occurs (table 4). The determination of plasma volume permitted the estimation of total circulating protein with the recognized minor discrepancy due to the presence of fibrinogen in plasma and its absence in serum. The increase in total circulating protein was significantly greater ($P = 0.01$) in pregnant animals than in non-pregnant controls. Therefore, what appears to be a reduction in total protein during pregnancy is only relative and there is, in reality, a significant absolute gain.

Beaton et al. ('53) found no difference between concentrations of serum protein

TABLE 4

Serum protein of pregnant and non-pregnant control and deoxypyridoxine-fed rats

	Total protein		Albumin		Alpha$_1$ globulin		Alpha$_2$ globulin		Beta globulin		Gamma globulin	
	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21
	gm%	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*
Non-pregnant												
Non-depleted, 0.8 mg% pyridoxine	7.08 ± 0.23[1]	7.98 ± 0.49	2.68 ± 0.19	3.04 ± 0.12	0.98 ± 0.15	1.28 ± 0.25	1.12 ± 0.06	1.11 ± 0.12	1.59 ± 0.04	1.59 ± 0.11	0.76 ± 0.06	0.83 ± 0.12
Depleted, 0.8 mg% pyridoxine	7.36 ± 0.42	7.79 ± 0.32	3.09 ± 0.28	3.07 ± 0.37	0.91 ± 0.10	1.02 ± 0.30	1.14 ± 0.14	1.05 ± 0.12	1.52 ± 0.11	1.74 ± 0.26	0.68 ± 0.11	0.91 ± 0.14
Depleted, 2.0 mg% deoxypyridoxine	7.40 ± 0.54	7.69 ± 0.48	2.97 ± 0.32	3.37 ± 0.26	1.00 ± 0.09	0.91 ± 0.28	1.29 ± 0.23	1.23 ± 0.17	1.59 ± 0.20	1.60 ± 0.15	0.65 ± 0.15	0.59 ± 0.12
	Total circulating											
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Non-depleted, 0.8 mg% pyridoxine	0.432 ± 0.029	0.492 ± 0.044	0.163 ± 0.007	0.189 ± 0.023	0.060 ± 0.011	0.078 ± 0.012	0.069 ± 0.007	0.069 ± 0.013	0.098 ± 0.008	0.100 ± 0.011	0.047 ± 0.004	0.051 ± 0.007
Depleted, 0.8 mg% pyridoxine	0.460 ± 0.058	0.476 ± 0.044	0.197 ± 0.028	0.189 ± 0.034	0.055 ± 0.006	0.062 ± 0.016	0.072 ± 0.011	0.063 ± 0.010	0.098 ± 0.014	0.108 ± 0.021	0.043 ± 0.011	0.054 ± 0.009
Depleted, 2.0 mg% deoxypyridoxine	0.420 ± 0.048	0.458 ± 0.061	0.166 ± 0.021	0.200 ± 0.025	0.056 ± 0.008	0.055 ± 0.019	0.072 ± 0.014	0.074 ± 0.015	0.069 ± 0.010	0.095 ± 0.011	0.037 ± 0.011	0.035 ± 0.009
Pregnant												
	gm%	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*	*gm%*
Non-depleted, 0.8 mg% pyridoxine	7.00 ± 0.43	6.29 ± 0.44	2.81 ± 0.34	2.56 ± 0.05	1.04 ± 0.09	1.08 ± 0.23	1.04 ± 0.18	0.99 ± 0.26	1.46 ± 0.08	1.20 ± 0.18	0.66 ± 0.14	0.47 ± 0.05
Depleted, 0.8 mg% pyridoxine	7.34 ± 0.44	6.88 ± 1.01	2.76 ± 0.23	2.79 ± 0.38	1.14 ± 0.25	1.06 ± 0.12	1.19 ± 0.17	1.00 ± 0.15	1.59 ± 0.20	1.41 ± 0.30	0.73 ± 0.10	0.63 ± 0.21
Depleted, 2.0 mg% deoxypyridoxine	7.15 ± 0.39	7.40 ± 0.41	2.90 ± 0.22	2.97 ± 0.24	1.10 ± 0.21	1.20 ± 0.22	1.05 ± 0.19	1.14 ± 0.15	1.52 ± 0.22	1.60 ± 0.15	0.62 ± 0.16	0.49 ± 0.13
	Total circulating											
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Non-depleted, 0.8 mg% pyridoxine	0.442 ± 0.064	0.505 ± 0.077	0.178 ± 0.033	0.205 ± 0.022	0.066 ± 0.012	0.085 ± 0.015	0.065 ± 0.010	0.081 ± 0.030	0.092 ± 0.012	0.097 ± 0.022	0.042 ± 0.012	0.038 ± 0.006
Depleted, 0.8 mg% pyridoxine	0.410 ± 0.043	0.566 ± 0.077	0.153 ± 0.023	0.230 ± 0.032	0.060 ± 0.009	0.088 ± 0.015	0.064 ± 0.012	0.083 ± 0.013	0.083 ± 0.008	0.116 ± 0.023	0.041 ± 0.008	0.050 ± 0.012
Depleted, 2.0 mg% deoxypyridoxine	0.428 ± 0.058	0.469 ± 0.060	0.174 ± 0.021	0.188 ± 0.028	0.066 ± 0.014	0.076 ± 0.017	0.063 ± 0.016	0.072 ± 0.010	0.092 ± 0.019	0.102 ± 0.018	0.038 ± 0.012	0.031 ± 0.007

[1] Standard error of the mean.

in vitamin B_6-deficient and control non-pregnant animals. The results of this study confirm this finding. However, although there was a slight increase in total protein concentration in the pregnant group fed deoxypyridoxine, the total circulating protein was significantly higher in pregnant animals fed pyridoxine ($P = 0.001$).

The concentrations of serum albumin were significantly lower ($P = 0.001$) at the end of the experimental period in all pregnant animals fed pyridoxine than in non-pregnant groups. Ross and Pike ('56) reported albumin concentrations in pregnant rats at term to be lower than those found in the literature for non-pregnant animals. In the pregnant groups in this study, however, albumin concentrations at term were not significantly different from those observed on the initial day of pregnancy. This observation is in contrast to that reported by Rinehart ('45) concerning the reduction of albumin concentration in normal pregnancy. In toxemia, he observed albumin levels to be even lower than in normal pregnancy. There was no greater reduction in albumin concentration in the deoxypyridoxine-fed animals in the present study (table 5). This may be associated with the fact that plasma volume did not increase in these animals to a level comparable with control groups.

Total circulating serum albumin, like total circulating serum protein, was significantly higher ($P = 0.001$) in the pregnant animals fed pyridoxine than in their non-pregnant controls, a further indication that blood levels expressed in terms of concentration are misleading when data for pregnant and non-pregnant animals are compared. Somewhat lower levels of total circulating albumin were found in the pregnant group fed deoxypyridoxine and these levels were similar to those of the non-pregnant groups. This is to be expected since plasma volume in these animals was more comparable to the non-pregnant controls. In the deoxypyridoxine-fed animals carrying litters, however, total circulating albumin levels were similar to those in pregnant controls.

Increases in the concentrations of alpha$_1$, alpha$_2$ and beta globulin fractions of serum have been observed in human pregnancy (Macy and Mack, '52). No sig-

TABLE 5

Serum protein of pregnant group fed deoxypyridoxine

	Total protein		Albumin		Alpha$_1$ globulin		Alpha$_2$ globulin		Beta globulin		Gamma globulin	
	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21
	gm %	*gm %*	*gm %*	*gm %*	*gm %*	*gm %*	*gm %*	*gm %*	*gm %*	*gm %*	*gm %*	*gm %*
With litters	7.27	7.07	2.97	2.91	0.88	1.18	1.16	1.04	1.68	1.50	0.77	0.44
	± 0.52[1]	± 0.24	± 0.10	± 0.23	± 0.13	± 0.19	± 0.16	± 0.08	± 0.26	± 0.16	± 0.09	± 0.06
Without litters	7.02	7.73	2.84	3.03	1.27	1.23	0.96	1.24	1.40	1.69	0.51	0.54
	± 0.11	± 0.23	± 0.27	± 0.22	± 0.05	± 0.24	± 0.16	± 0.14	± 0.05	± 0.06	± 0.11	± 0.15
					Total circulating serum protein							
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
With litters	0.433	0.493	0.179	0.202	0.052	0.082	0.070	0.072	0.102	0.106	0.047	0.031
	± 0.071	± 0.071	± 0.029	± 0.029	± 0.001	± 0.018	± 0.016	± 0.009	± 0.024	± 0.023	± 0.010	± 0.007
Without litters	0.422	0.445	0.170	0.175	0.076	0.070	0.058	0.071	0.085	0.093	0.031	0.031
	± 0.041	± 0.033	± 0.012	± 0.019	± 0.009	± 0.014	± 0.013	± 0.011	± 0.010	± 0.008	± 0.009	± 0.008

[1] Standard error of the mean.

nificant increases in alpha$_1$ or alpha$_2$ globulin concentrations were observed in the pregnant rats in this study and beta globulin was significantly decreased ($P = 0.001$) in the pregnant animals. However, total circulating levels of all three fractions were increased.

Concentrations of alpha$_1$ globulin were increased in non-pregnant controls ($P = 0.001$), and significant increases in total circulating alpha$_1$ globulin were observed in all of the pyridoxine-fed animals ($P = 0.01$). Other members of the vitamin B-complex, folic acid and vitamin B_{12}, also have been shown to influence alpha$_1$ globulin concentration (Mulgaonkar and Sreenivasan, '57).

The most significant change in the serum protein, however, occurred in the gamma globulin fraction. The reduction in gamma globulin found in the pregnant animals ($P = 0.001$) is similar to that reported for the pregnant human (Macy and Mack, '52). Total circulating gamma globulin also tended to be somewhat lower. These data suggest that the reduction in gamma globulin is a true effect of pregnancy and is independent of changes in plasma volume. Beach et al. ('52) have observed lower concentrations of gamma globulin in maternal blood than in cord blood. Since gamma globulin is known to pass through the placenta to the fetus (Hughes, '54), it seems likely that the reduction in gamma globulin, like the increase in blood volume, is a normal physiological response to pregnancy.

Low concentrations of gamma globulin and total circulating gamma globulin were observed in animals maintained on deoxypyridoxine. This finding may be associated with the inability of pyridoxine-deficient rats to produce circulating antibodies in response to injections of various antigens (Axelrod et al., '47). When depleted animals were fed pyridoxine during the experimental period, increases in gamma globulin concentration were observed on the final day of experiment. These data suggest that vitamin B_6 plays a role in the synthesis of gamma globulin.

SUMMARY AND CONCLUSIONS

Changes in blood volume, hemoglobin and serum protein have been investigated in deoxypyridoxine-fed pregnant and non-pregnant rats and in control rats fed pyridoxine under similar conditions.

Total blood volume increased significantly in pregnant animals fed pyridoxine. There was a slight increase in total blood volume in deficient animals carrying young. Decreases in hematocrit and hemoglobin levels appeared to be associated with hemodilution in control pregnant animals but apparently were more directly attributable to the effect of the antagonist in deoxypyridoxine-fed animals. Support for this conclusion is drawn from the observation that the absolute amount of circulating hemoglobin as determined from total blood volume was not diminished in control pregnant rats, whereas, both the concentration and absolute quantity of hemoglobin were reduced in the deoxypyridoxine-fed animals.

Concentrations of serum total protein and albumin were lower in pregnant animals, but the total circulating protein and albumin were higher in pregnant than in non-pregnant animals. Gamma globulin concentrations and total circulating gamma globulin were reduced in pregnant animals and in deoxypyridoxine-fed non-pregnant animals.

Changes in blood volume, hemoglobin and serum protein in the deoxpyridoxine-fed animals have been compared with those reported for human toxemia of pregnancy. Although the physiological response of the pregnant rats fed deoxypyridoxine was altered from that of controls fed pyridoxine and on this basis may be considered abnormal, there were no clear-cut similarities between the response of the deoxypyridoxine-fed animals and the toxemic syndrome as it occurs in the human.

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Start each of the following sections on a new page and typed double-spaced:

1. Tables, with appropriate heading (a separate sheet for each table)
2. Explanation of figures, numbered and listed consecutively
3. Literature cited, arranged alphabetically by authors—see current issue for exact form, and
4. Footnotes.

Footnotes. In the text, footnotes should be numbered consecutively, including any indicated on page 1. In a table, footnotes should be made part of the table, typed directly beneath it, and numbered 1, 2, 3, etc. These are independent of other footnote numbers used in the manscript.

Acknowledgments. Financial support should be listed as a footnote to the title. Credit for materials should be listed as a footnote in the text. Technical assistance and advice may be cited in a section headed Acknowledgments, which will follow the summary.

Summary. This should represent a brief recapitulation of the problem and results.

Literature Cited. Accuracy and adequacy of the references are the responsibility of the author. Literature cited should be checked carefully with the original publication. References to abstracts of verbal reports, personal communications or unedited reports should be treated as text footnotes. Reference to a paper in press may be included in Literature Cited. If such a paper would be useful in the evalua-

tion of the manuscript under consideration, it is advisable to make a copy of it available to the Editor. When a manuscript is one of a series of papers, the preceding paper should be included in Literature Cited.

Numbers. If possible, avoid using a number as the first word in a sentence. Numbers so used should always be spelled out, but no abbreviations should follow such numbers. Numbers indicating time, weight and measurements are to be expressed in Arabic figures when followed by an abbreviation (e.g., 2 mm; 1 sec.; 3 gm; 100 mm^3). Except as indicated, the numbers *one, two* and *three* will be spelled out in the text. All higher numbers will be given in Arabic figures. Use Arabic numerals throughout, including tables.

Abbreviations. The word *figure,* written in the text, is not abbreviated except when appearing in parentheses, as (fig. 2) (figs. 4–6).

The words *pounds, weeks* and *days* are not abbreviated.

The metric system should be used in preference to others for measurements, weights, etc. Temperatures should always be expressed in Centigrade scale. Metric abbreviations listed below are shown in the approved lower-case style, *without periods:*

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
m^3	cubic meter	μμ	micromicron
Volume		*Area*	
cm^3	cubic centimeter		
mm^3	cubic millimeter	m^2	square meter
l	liter	cm^2	square centimeter
ml	milliliter	mm^2	square millimeter

Symbols. When preceded by a figure, the following symbols are used:

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

Tables and figures. Follow form in current issues for the use of upper and lower case letters and italics. Authors are urged to economize on space used for tables and figures. These should fit one column width (2⅝ inches) or when necessary, two column widths (5½ inches). A charge will be made for that space used for tables and figures which exceeds one-half of the space used for the manuscript exclusive of tables and figures. A table or figure should be constructed so as to be intelligible without reference to the text. Lengthy tabulation of essentially similar data can often be avoided by giving the number of experimental results and their mean values, with standard deviations or ranges within which the values fall. Statements that significant differences exist between the mean values of two groups of data should be accompanied by indications of probability derived from the test of significance applied.

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Figures should be lettered in *black* India ink. For any charts made on cross-section paper, use India ink on paper printed in light blue only. Charts drawn in India ink should be so executed throughout, with no typewritten material included. Graphs and charts should be given consecutive figure numbers as they will appear in the text.

American Documentation Institute. To conserve space in the Journal, lengthy descriptions of experimental procedures, detailed data such as that on individual animals or subjects, extra figures and other material considered important supplementary information, may be deposited with the American Documentation Institute, Library of Congress, through the Editor's office with a footnote reference.

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ERRATUM

Cremer, H. D., H. J. Kinkel and J. Mauron 1960 Dental caries and growth in rats fed whole-milk powders with increasing lysine deterioration. J. Nutrition, *70:* 533. Table 1, footnote 3, should have read as follows: Difference between amount of lysine liberated enzymatically from fresh milk and that freed from the dried milk, expressed in per cent of lysine liberated from fresh milk.

To correct footnote 3 in your copy of volume 70, number 4, please cut along lines of reprinted footnote section below and paste over the footnotes on bottom of page 534.

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[1] Difference between lysine content of fresh milk and that of the sample, determined after acid hydrolysis expressed in per cent of lysine content of fresh milk.

[2] Difference between lysine deterioration and destruction.

[3] Difference between amount of lysine liberated enzymatically from fresh milk and that freed from the dried milk, expressed in per cent of lysine liberated from fresh milk.

[4] Lysine freed enzymatically from the sample expressed in per cent of lysine liberated from fresh milk.

A LABORATORY ATLAS

OF THE 13-MM PIG EMBRYO

(Prefaced by younger stages of the chick embryo)

by

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Professor of Anatomy
University of Minnesota

THIRD EDITION
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